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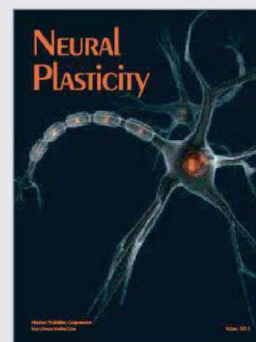
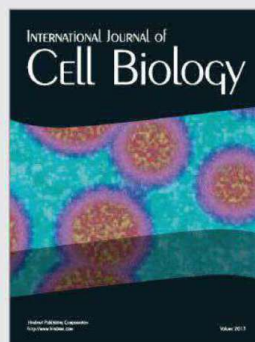
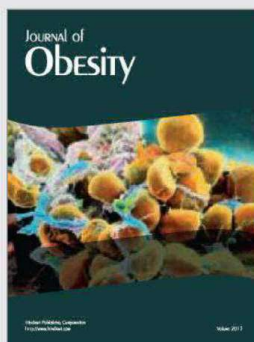
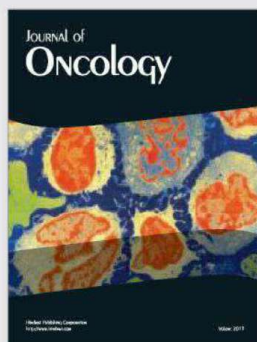
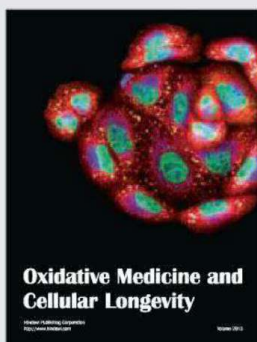
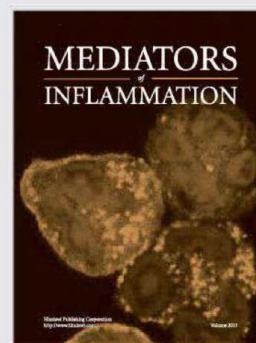
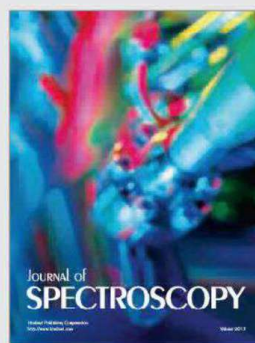
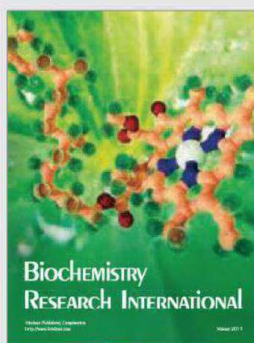
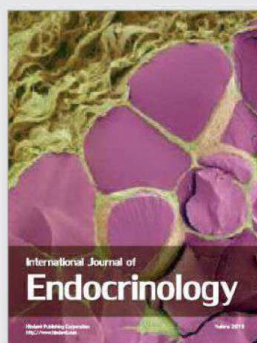
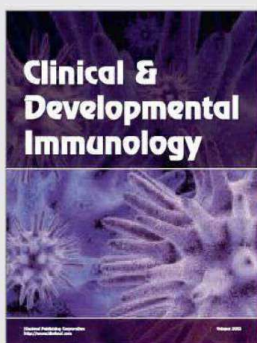
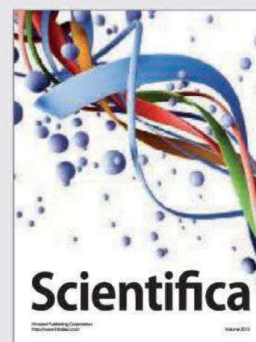
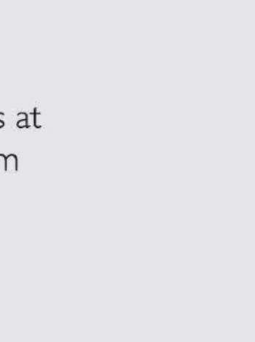
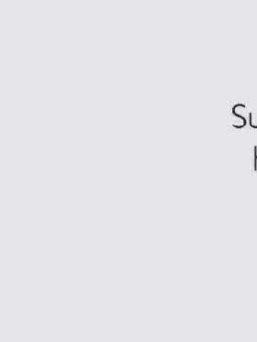
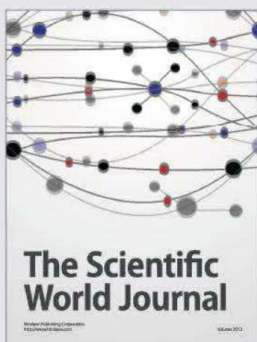
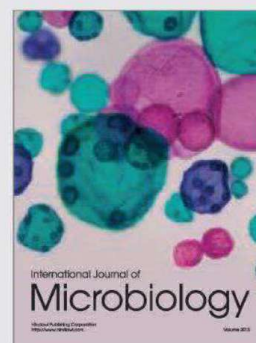
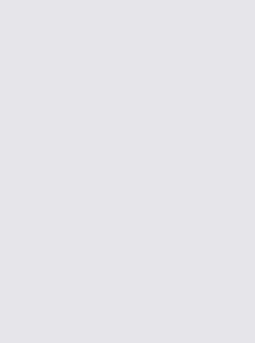
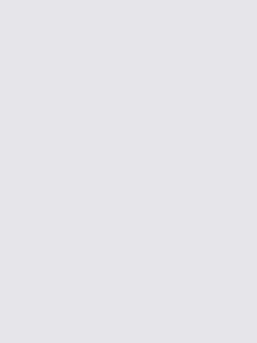
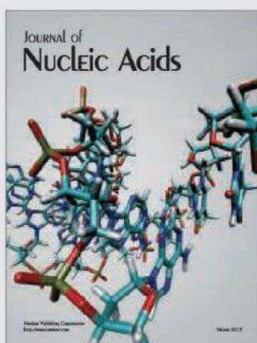
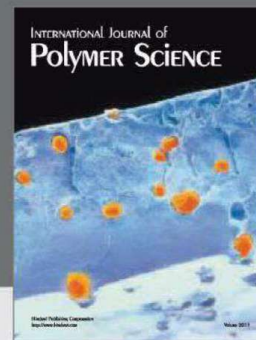
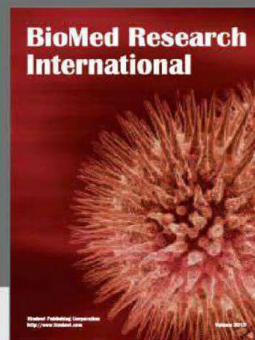
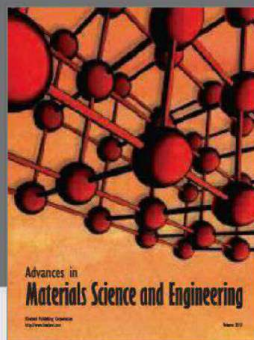
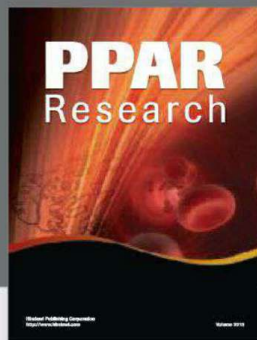
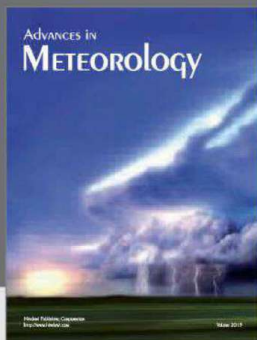
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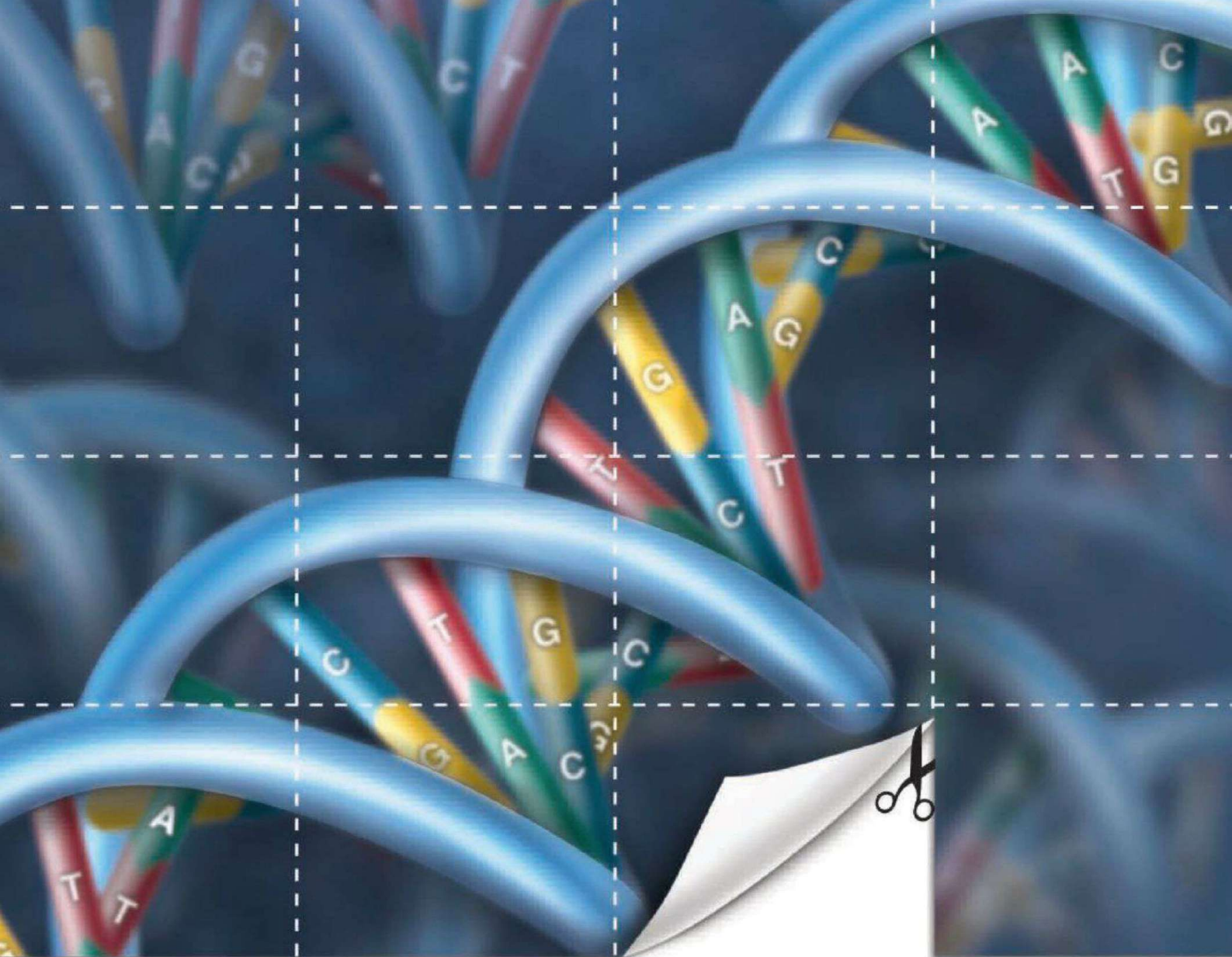
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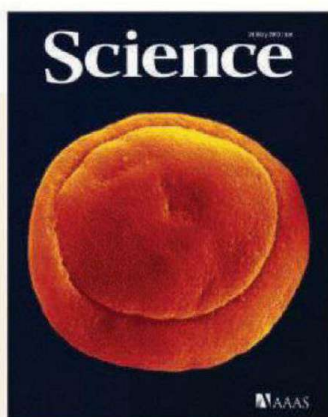


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ON THE WEB THIS WEEK

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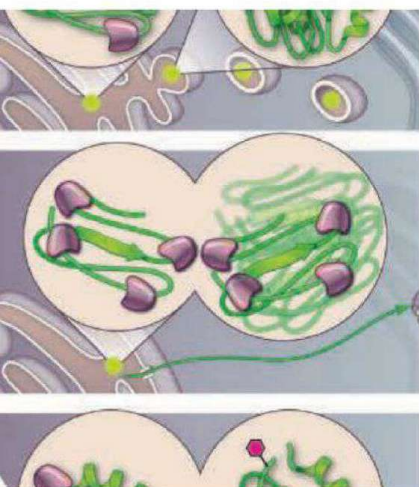
COVER

A *Classopollis meyeriana* conifer pollen grain (~30 micrometers across) preserved in lake sediments deposited just after a massive, volcano-induced extinction event ~200 million years ago. This fossilized pollen grain is representative of the low-diversity plant assemblages found within the tropics of the Pangean supercontinent during the postextinction recovery period. See page 941.

Image: Dee Breger and Sarah Fowell/Science Source

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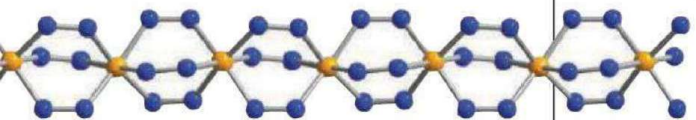
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Life Versus the Volcanoes

Correlating a specific triggering event, such as an asteroid impact or massive volcanism, to mass extinction events is clouded by the difficulty in precisely timing their occurrence in the geologic record. Based on rock samples collected in North America and Morocco, **Blackburn et al.** (p. 941, published online 21 March) acquired accurate ages for events surrounding the mass extinction that occurred ~201 million years ago, between the Triassic and Jurassic Periods. The timing of the disappearance of marine and land fossils and geochemical evidence of the sequential eruption of the Central Atlantic Magmatic Province imply a strong causal relationship.

Telling Hexanes Apart

The efficiency of modern internal combustion engines depends on the relative reactivity of the hydrocarbons that comprise the fuel. In particular, branched hydrocarbons are less likely than their linear counterparts to react prematurely—



a property reflected in the fuel mixture's octane number. **Herm et al.** (p. 960) report a metal organic framework material with triangular pore channels that discriminate among the differently shaped isomers of hexane more finely than the commercial standard.

For Good Measure

SS Cygni is a well-studied binary star system in the northern constellation Cygnus, consisting of a white dwarf that accretes matter from its companion star. **Miller-Jones et al.** (p. 950; see the Perspective by **Schreiber**) used radio observations to derive a model-independent distance to this prototypical accreting white dwarf system. The measurement places the system significantly closer than previously determined, reconciling the observed properties of SS Cygni with our current understanding of accretion theory.

A Touchy Subject

The ability to hold a glass being filled with water without dropping it depends on our ability to touch objects and to know the correct pressure to exert. Thus, for robotics or artificial skin design, methods are needed for sensitive pressure detection. **Wu et al.** (p. 952, published online 25 April) designed a device based on an array of

zinc oxide nanowires that generate a small voltage when flexed that could be translated into a pressure signal. The device has a pressure-sensing range of up to 30 kPa, comparable to the 10 to 40 kPa range of a human finger.

The Master Switch for Itch?

Recently, gastrin-releasing peptide (GRP) has been implicated as the primary neurotransmitter between itch-sensitive nerve fibers and downstream neurons in the spinal cord. However, **Mishra and Hoon** (p. 968) challenge this view, provide evidence that natriuretic polypeptide b (Nppb) is the central itch neurotransmitter, and suggest that GRP is released by second-order neurons in the spinal dorsal horn that express the Nppb receptor and are excited by Nppb.

Spleen Knockout Explained

Isolated congenital asplenia (ICA) is a rare disorder where patients are born without a spleen and are at increased risk of bacterial infection but have no other developmental abnormalities.

Through sequence analysis of familial and sporadic cases, **Bolze et al.** (p. 976, published online 11 April) found that ICA patients carry mutations in the gene encoding ribosomal protein SA and as a

result express about half the normal amount of this protein. The mechanism by which reduced expression of a housekeeping protein causes an organ-specific defect remains unclear.

Folding Too Slow, Off You Go

One of the major questions of chaperone-assisted protein-folding pathways is how substrates that fail to fold avoid futile folding cycles. **Xu et al.** (p. 978; see the Perspective by **Kleizen and Braakman**) developed a model to examine a folding-competent protein that nevertheless fails to fold within the endoplasmic reticulum. Under these circumstances, the unfolded protein was subject to an unusual glycosylation, O-mannosylation, which appeared to terminate folding of the unfinished molecules. Eliminating O-mannosylation allowed the protein to fold completely.

Malaria Cloak and Dagger

Mosquitoes have a complex immune system capable of effective antiparasite responses; however, malaria transmission is still highly efficient. **Molina-Cruz et al.** (p. 984, published online 9 May; see the Perspective by **Philip and Waters**)



Sugar Aversion

Several populations of the German cockroach have become averse to the glucose used as bait in toxic traps, which has severely reduced the traps' effectiveness. **Wada-Katsumata et al.** (p. 972; see the cover) show that this aversion is a result of changes in the peripheral gustatory system, whereby glucose, as well as "sweet" receptors, stimulated an aversive bitter compound receptor.

show that *Plasmodium falciparum* has a gene product, *Pfs47*, that makes the parasite's ookinetes "invisible" to the mosquito immune system. Disruption of this mechanism could potentially be used to block malaria transmission.

Sensing Tension

Many cellular processes are regulated by mechanical signals. Single-molecule force spectroscopy has been used to probe molecular unfolding or unbinding; however, measuring the single-molecule forces required to activate signaling remains a challenge. **Wang and Ha** (p. 991) describe a "Tension Gauge Tether" approach to measure the force applied on a single receptor ligand bond. By using tethers with a range of tension tolerances and monitoring activation, the force required to activate signaling could be measured.

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Colin Norman has been News Editor of *Science* since 1995.

A Fond Farewell

HALF A CENTURY AGO, PHILIP ABELSON, *SCIENCE*'S LONGTIME EDITOR-IN-CHIEF, MADE A RADICAL move in the world of scholarly publishing. He launched a new section of the journal, News and Comment, and staffed it with journalists, not scientists. His rationale: Fundamental changes were taking place in the funding and organization of research, and it was becoming increasingly important for scientists to know about and understand events shaping the scientific enterprise. A decade later, he added another journalist-written section, Research News, that would report on new findings and trends in research itself. This time, he reasoned that because research was becoming ever more specialized, scientists needed objective reporting on developments in disciplines outside their own. Under the leadership of early News editors such as Daniel S. Greenberg and Allen Hammond, the News sections quickly became among the most widely read parts of a journal that has continued to publish the very best research papers.

I believe that Abelson's reasoning is as sound today as it was 50 years ago. The need has never been greater for good, timely reporting on an enterprise that is now global and is widely viewed as a critical element in economic development and in the solution of many societal problems. It is also an enterprise pushed and pulled by internal and external forces, filled with larger-than-life characters, and engaged in some of the most exciting and intellectually challenging pursuits imaginable. In short, it's a reporter's dream. And in a world in which information is increasingly obtained by targeted searches, the disciplinary narrowing that Abelson lamented is growing more pronounced, and it is happening at a time when advances are most likely to be made at the interfaces between the sciences. The merger of computer science and biology and the resulting explosion of "-omics" findings is just one example of that promise.

It has been my privilege to have been part of *Science*'s News team for more than 30 years, the past 18 as News Editor, and to have served under the leadership of five distinguished Editors-in-Chief, beginning with Phil Abelson. Those decades have seen dramatic changes in our operation. We have gone from a solely Washington, D.C.-based unit to one with bureaus in China, Japan, India, Germany, the Netherlands, France, the United Kingdom, and four other U.S. cities, as well as a stable of freelance writers who report from many other places. Our readers are no longer restricted to those who have access to a print copy; we have extended our reach to anybody who has access to the Internet, with a free daily news service consisting of *ScienceNOW* and *ScienceInsider* that has more than a quarter of a million Facebook

fans and that, in many months, attracts upward of 1 million unique visitors. In addition, through the journal's Web site, our reporting, analysis, and feature writing that are produced for the weekly print magazine reach a growing international audience. As a mark of excellence, our reporters have received some two dozen science writing awards in the past decade.

If this essay sounds like the reminiscences of somebody who is about to depart, it is. Next month, I will be stepping down as *Science*'s News Editor and handing over the reins to Tim Appenzeller, an outstanding and seasoned science journalist. Tim was an editor at *The Sciences* and *Scientific American* before joining *Science*'s News team as a Deputy News Editor in the 1990s. Since then he has been a senior editor at *U.S. News and World Report* and *National Geographic*, and for the past 3 years he has been *Nature*'s Chief Magazine Editor. It is very gratifying to know that *Science*'s News department and its exceptional team of reporters and editors will be in such capable hands, as *Science* and science continue to undergo changes that promise to be every bit as dramatic and exciting as those of the past several years.



Online

sciencemag.org

S Podcast interview
with author Colin
Norman (http://scim.ag/ed_6135).

— Colin Norman

10.1126/science.1240454

ENGINEERING

Lithium Batteries in the Afterlife

We are accustomed to collecting single-use batteries for recycling, but what about rechargeable lithium batteries in consumer electronic products? Many of these products are disposed of in the normal rubbish with the batteries still inside. The harmful nature of this e-waste has been recognized, but previous studies have focused on the impacts of the electronic products—such as cellphones—themselves, rather than the batteries contained in them. Kang *et al.* have performed leaching tests and applied life-cycle impact and hazard assessment models to determine the environmental impacts and human toxicity potentials of lithium-ion batteries used in cell phones. They analyzed 16 batteries from cell phones sent for recycling, representing the three types of lithium battery that are most abundant in e-waste: lithium-ion and lithium-polymer from traditional phones and small, high-energy-density lithium batteries from smartphones. The authors show that these batteries contain several metals, including lead and cobalt, that can leach out under simulated landfill conditions at concentrations exceeding U.S. federal and state regulations. They call for regulatory efforts to increase recycling and reuse of rechargeable batteries and to reduce the levels of toxic metals in these batteries and in consumer electronics as a whole. — JFU

Environ. Sci. Technol. **47**, 10.1021/es400614y (2013).



CELL BIOLOGY

Little and Large

The import of proteins into the nucleus occurs through pores in the nuclear envelope and depends on the Ran GTPase system. Previous work has shown that the nuclear import of the protein Tpr (translocated promoter region) is compromised in fibroblasts that express the Progerin protein associated with Hutchinson-Gilford progeria syndrome (HGPS) (below; see bottom row, purple). These findings were attributed to deficiencies in the Ran GTPase system, whereby Ran GTP accumulates in the nucleus to promote the release of nuclear import cargoes from their receptors. Following up on these findings,

nucleoporin that forms a basketlike structure on the nuclear side of nuclear pores. Its import into the nucleus is particularly sensitive to the Ran gradient across the nuclear envelope. The extent to which problems with Tpr import or the import of other large protein cargoes contributes to the premature aging phenotypes observed in HGPS patients remains unclear. — SMH

J. Cell Biol. 10.1083/jcb.201212117 (2013).

PSYCHOLOGY

Lean In for Equality

The importance of collaborative groups is being promoted in many school systems, and increasingly, scientific progress is made by

groups of scientists rather than individual efforts. How do women perform in collaborations? Haynes and Heilman examined how gender stereotypes and self-perceptions manifest in collaborations in experiments with students from an introductory psychology class. Participants could not see their “partner” (whose description and performance were created by the experimenters). In the first experiment, participants either received feedback about

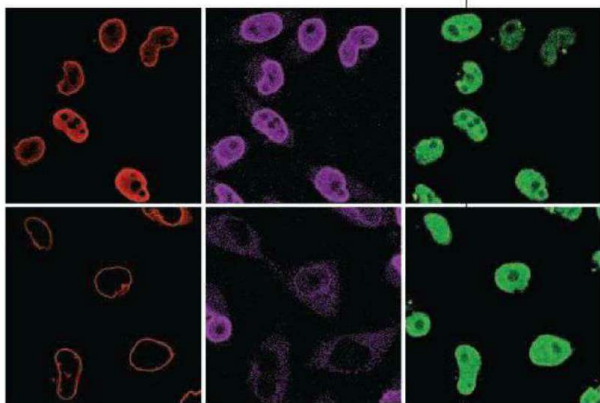
This was observed regardless of whether participants worked on the task as a unit or each had their own components to fulfill. However, this was affected by the gender of the “partner”: Women who thought their partner was male tended to devalue their own contributions but did not devalue them if they thought they had collaborated with another woman. These results suggest that women tend to minimize their own contributions, and they indicate that this is an additional obstacle that must be overcome in efforts to gain gender equality in the workplace. — BJ

Pers. Soc. Psychol. Bull. 10.1177/0146167213486358 (2013).

BIOCHEMISTRY

A Turn-On for Kinases

Understanding of cellular regulatory mechanisms can be hindered by the inability to precisely control the activity of individual signaling components. Dagliyan *et al.* thus set out to create engineered protein kinases that could be activated by an exogenous ligand. They used the ligand-dependent protein-protein interaction between FKBP12 (12-kD FK506-binding protein) and FRB (FKBP12-rapamycin binding protein) that occurs in the presence of rapamycin. The goal was to confer ligand-dependent regulation in a manner that was generalizable to other proteins. Through a combination of rational design and trial and error, they were able to make a conformational switch module that allowed such control of several different



Snow *et al.* now show that, rather than a global reduction in nuclear import, the limitations on Tpr import reflect difficulties in the import of large protein cargoes into the nucleus. Tpr is a

the performance of their group as a whole or received feedback about their own performance. Unless given specific feedback, women tended to undervalue their own contributions.

protein kinases. Such a strategy is expected to help alleviate the challenges of elucidating the roles of individual components of complex cell regulatory circuits in vivo. — LBR

Proc. Natl. Acad. Sci. U.S.A. **110**, 6800 (2013).

EDUCATION

Drawing to Learn

Science education is shifting away from the memorization of facts and moving toward educational experiences that correspond with authentic research and the scientific process. Although this culture shift has resulted in gains being made in research on scientific literacy, there remains little published work on imaginative practice traits or how scientists learn to grasp and convey microscopically small subject matter. To investigate this learning system further, Hay *et al.* asked undergraduates, trainee scientists consisting of Ph.D. students and postdocs, and leading neuroscience researchers to "draw a neuron." Using employed qualitative analysis, drawing-sorting exercises, and hierarchical cluster analysis, the team looked for categorical differences in the drawings and whether any existing differences could be related to the levels of research experience of the participants. Compared to drawings from seasoned researchers and trainee scientists, undergraduate drawings looked remarkably like textbook reproductions, which suggests that years of previous teaching had convinced these students that textbooks are authoritative. The research team employed two different teaching interventions, designed for students to directly experience "life as a neuron," and upon completion students were again asked to "draw a neuron." Post-intervention drawings by students were found to be similar to experts' drawings, suggesting that interventions such as these have great potential to increase undergraduates' creative aptitude when it comes to science education. — MM

Sci. Educ. 10.1002/sce.21055 (2013).

GENETICS

Tagging New Genes

Epialleles are heritable, nongenetic (epigenetic) differences in DNA methylation. Although

variation among epialleles in the model plant *Arabidopsis thaliana* has been observed in laboratory populations, the frequency and effects of epialleles in natural populations are largely unknown. Silveira *et al.* examined epialleles of the *Qua-Quine Starch (QQS)* gene, which is unique to *Arabidopsis* and is believed to have evolved recently. *QQS* gene expression demonstrated epigenetic control. *QQS* expression correlated negatively with the degree of methylation at the promoter and varied among global accessions and wild populations. Thus, variation in gene expression via differentially regulated methylation in wild populations may be a mechanism for allowing selection to act on the expression levels of *QQS* and other evolutionarily de novo genes. Regions in the genome may undergo spontaneous changes in their epigenetic status, and these differences may be one evolutionary mechanism affecting the evolution of new genes. — LMZ

PLoS Genet. **9**, e1003437 (2013).

PHYSICS

Wedging in Plasmons

Although light provides the capability of transferring vast amounts of data quickly, nanoelectronics offer the ultimate in shrinkability for chip-scale circuits. Integrating or hybridizing the two technologies is desirable but challenging because of the several orders of magnitude difference in length scales between them. Surface plasmons, the light-induced collective electronic excitations that propagate at the surface of a metal, may offer the possibility of a robust bridge. Much effort, therefore, is being directed to manipulating the direction and lifetime of these propagating excitations. Using a graded index wedge of photoresist on top of a layer of gold, Bleckmann *et al.* show that the propagation of beams of nondiffracting (nonspreading) surface plasmons along the gold surface is dependent on the geometrical parameters of the wedge-shaped dielectric. The results suggest a fairly simple route to the launch and control of the directional propagation of surface plasmons for future nanoscale optoelectronic circuits. — ISO

Opt. Lett. **38**, 1443 (2013).

CHEMBRIDGE: A MAJOR FORCE IN BUILDING BLOCKS

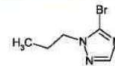
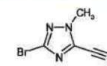
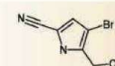
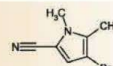
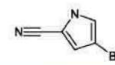
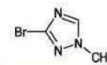
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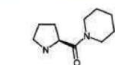
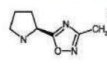
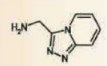
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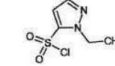
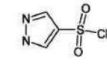
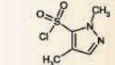
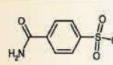
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AROUND THE WORLD



Kiruna, Sweden 1

Asian Nations Join Arctic Council

Five Asian countries, including China and India, gained observer status to the Arctic Council at last week's semiannual ministerial meeting. The eight nations with Arctic territory, including the United States, are the only members of the council with decision-making powers, and a number of indigenous groups are permanent participants that can address the council. But many other organizations and countries are nonvoting observers in the primary diplomatic organization determining policy in the region. With predictions of a summertime ice-free route through Arctic waters within a decade, China and others had lobbied

hard for a seat at the table as observers.

Following dissent from Canada, however, the council deferred a decision on an observer application from the European Union, possibly due to ongoing disputes over an E.U. ban on seal fur and proposed restrictions on imports of oil produced in the Alberta tar sands.

Washington, D.C. 2

Kepler's Closing Act?

One of the most successful missions in NASA history may be drawing to a close. NASA officials announced on 15 May that the Kepler spacecraft, which monitors 150,000 sunlike stars in search of transiting planets—and has found more

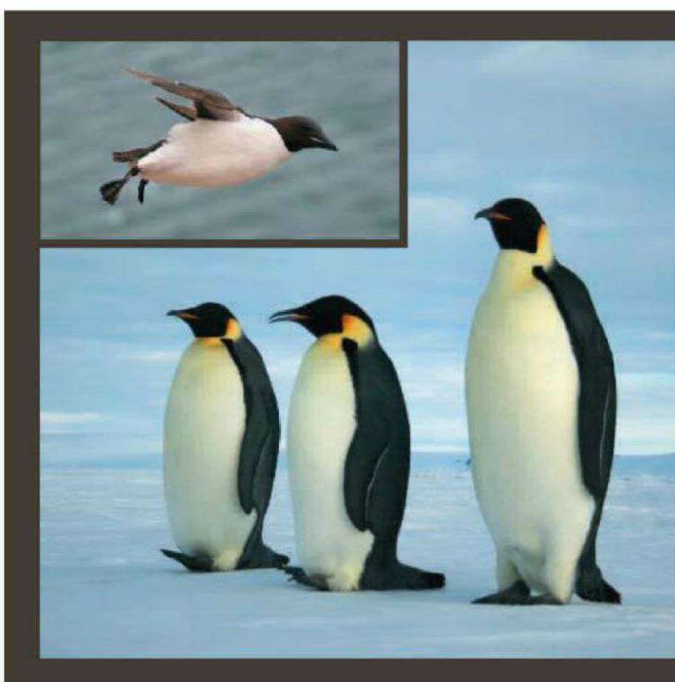


than 2700 planetary candidates outside the solar system—can no longer point in a specified direction due to the malfunctioning of one of its reaction wheels. The spacecraft was put into safe mode while engineers work on the problem.

Launched in 2009, the Kepler mission completed its 3.5-year planned run last year, beginning an extended 3.5-year mission in November. Officials were hopeful that it would continue beaming back data until 2016. But that now looks uncertain; the spacecraft needs three reaction wheels to point precisely, and another wheel had already failed last year.

Still, “we are not down and out,” says Charles Sobeck, deputy project manager for Kepler at NASA's Ames Research Center in Moffett Field, California. “The spacecraft is safe and stable. We'll proceed with our investigation.” Sobeck says that “the mission itself has been spectacularly successful. We have lots of data on the ground still to pore through.”

<http://scim.ag/Keplerend>



Flight of the Penguin

More than 70 million years ago, the ancestors of penguins could soar through the air. So why did the penguin give up flight? Scientists have now confirmed a long-suspected answer: It would rather swim.

Being both a diver and a flyer is costly and inefficient, suggests a new study of murres, penguinlike seabirds that both swim and retain command of the air. Murres “are awful flyers,” says graduate student Kyle Elliott of the University of Manitoba in Winnipeg, Canada, an author of the new paper published online this week in the *Proceedings of the National Academy of Sciences*. “They beat their wings really, really fast, and they're horrible at landing.”

Thick-billed murres (*Uria lomvia*, inset), which nest on cliffs in Alaska, Canada, and other northerly sites, expend more energy per minute of flight than any other bird, the team found. On the wing, murres burn energy at 31 times their rate at rest, the highest known ratio in a bird. In water, they're more efficient than many birds, but expend more energy while diving when compared with penguins. That suggests that by giving up flight, penguins improved their diving prowess.

CREDITS (TOP TO BOTTOM): NASA/AMES WENDY STENZEL; © LIN PADGHAM; (INSET) KYLE H. ELLIOTT

By the Numbers

30th Anniversary of the discovery of the AIDS virus. Unveiled in *Science* on 20 May 1983, the discovery earned Institut Pasteur researchers Luc Montagnier and Françoise Barré-Sinoussi Nobel prizes.

25 Cubic kilometers of ground water pumped by the United States each year from 2000 to 2008, according to the U.S. Geological Survey. The average rate over the past century was 9.2.

194,000 Number of signatures on a change.org petition to drop charges against Florida teen Kiera Wilmot, who was arrested on 1 May for causing a small explosion at her school during a science experiment. Charges were dropped on 15 May.

Shanghai, China 3**H7N9 Hazard Particularly High**

The risk that the H7N9 avian influenza virus poses to humans is “unusually serious,” noted a joint World Health Organization (WHO) and Chinese National Health and Family Planning Commission report. The report said the possibility that the virus could become transmissible among people is “higher than for any other known avian influenza virus.”

The document, which was posted 18 May on the WHO website, grew out of a weeklong investigative mission in late April by four international scientists. The team surveyed Chinese poultry markets and labs to evaluate the severity of the H7N9 outbreak, which infected 131 people by

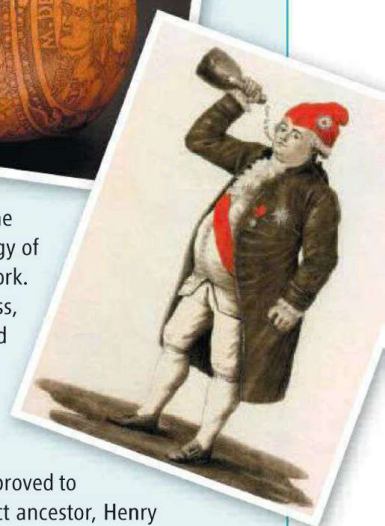
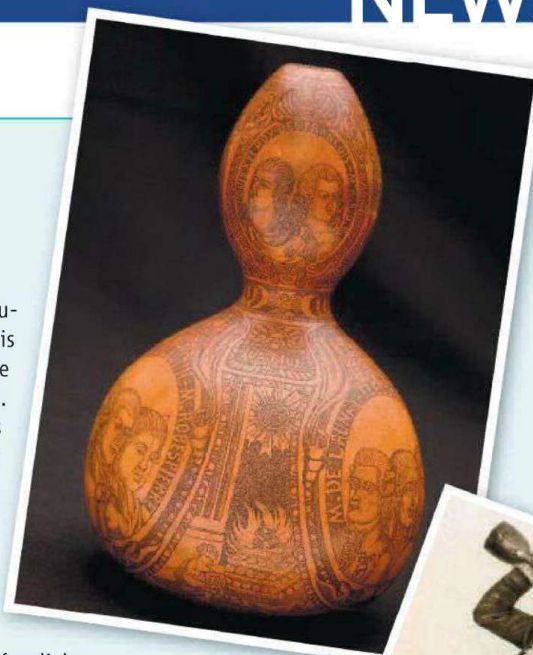
Random Sample**Was the Downfall of Louis XVI in His DNA?**

In 1793, as the French Revolution reached its height, King Louis XVI and his wife Marie Antoinette met their fates at the guillotine. According to legend, a witness soaked up the king's blood in a handkerchief and stored the cloth in an engraved gourd.

Turns out, the legend was true. DNA from that gourd shows not only that the blood was the king's but also that Louis carried genetic risk factors for diabetes, obesity, and bipolar disorder, reported Carles Lalueza-Fox of the Institute of Evolutionary Biology in Barcelona, Spain, at the Biology of Genomes meeting earlier this month in Cold Spring Harbor, New York. Some historians have blamed Louis's downfall on his indecisiveness, but the genome suggests that the king's inept rule might have had a biological basis, Lalueza-Fox said.

There was scant human DNA in the gourd; most of the DNA came from the squash or from bacteria associated with rotting vegetables. At first, all Lalueza-Fox could say was that the human DNA was from a blue-eyed European male. But the Y chromosome proved to match one obtained from the mummified head of the king's direct ancestor, Henry IV of France. That head has its own odd history: Lost after looters ransacked royal graves during the French Revolution, it was recovered and identified in 2010.

The findings are a bellwether, says Jeffrey Kidd, a population geneticist at the University of Michigan, Ann Arbor. “The combination of these approaches to recover information from small amounts of degraded DNA is going to open up all sorts of fascinating history stories,” he says.



17 May, killing 36, according to WHO. The report recommends surveillance through the summer, although new cases have dropped off.

FINDINGS**Combining Cancer Immunotherapy Drugs Shows Promise**

Cancer drugs that harness the body's immune system to destroy tumors may work better when two drugs are combined, a small clinical trial suggests.

The study combined a drug approved in 2011 for advanced melanoma, ipilimumab, with an experimental drug called nivolumab. Ipilimumab blocks a protein called CTLA-4 and nivolumab blocks the PD-1 protein; tumors use both proteins to hide from the immune system. Forty percent of 52 patients responded to the drug combination. Nearly

one-third saw their tumors shrink by at least 80% within 3 months—a rate higher than had been seen with either drug alone. Some patients' tumors have been kept in check for a year or more.

“It was really the rapidity and the magnitude of the response that was impressive to us,” said Jedd Wolchok of the Memorial Sloan-Kettering Cancer Center in New York City, who will present the study in early June in Chicago at the annual meeting of the American Society of Clinical Oncology. Bristol-Myers Squibb, which makes both drugs, now plans to test them in a larger trial.

Science LIVE

Join us on Thursday, 30 May, at 3 p.m. EDT for a chat on the **ethics of studying chimps in captivity**. <http://scim.ag/science-live>

NOTED

>The OSIRIS-REx mission **to return a bit of asteroid to Earth** got NASA's final approval last week. Set to launch in 2016, the mission will return 60 grams of 490-meter-diameter Bennu in 2023. That puts the \$1 billion robotic mission ahead of NASA's unfunded plan for a robotic spacecraft that would snag a 500-tonne asteroid and haul it to Earth orbit no earlier than 2022 for astronauts to study.

EPIDEMIOLOGY

Report Reignites Battle Over Low-Salt Diets

Radically reducing your salt intake is good for your health. Or is it? That question is at the center of a passionate debate once again after an Institute of Medicine (IOM) report last week called into question official recommendations advising people to consume less sodium. The high-profile study is the latest in a controversy that has raged for decades—and some researchers say the science of salt is so complex that they may never find solid answers.

U.S. dietary guidelines recommend a daily intake of less than 2300 mg of sodium—equivalent to 6 grams, or one teaspoon, of salt—for the general population, and less than 1500 mg for people at an increased risk of cardiovascular disease. The latter includes African Americans, people with high blood pressure or kidney disease, and anyone over the age of 50—in other words, about half of the U.S. population. The American Heart Association (AHA) goes even further; it says that everybody should stick to the lower limit.

But an expert panel convened by IOM says that there is little evidence that dropping below 2300 mg of salt lowers the risk of cardiovascular disease—and some hints it might actually do harm. AHA immediately criticized the study, calling it “incomplete.” Elliott Antman, an expert on cardiovascular diseases at Brigham and Women’s Hospital in Boston, chides the panel for overplaying “very flawed data” and warns the report could harm public health. But epidemiologist Michael Alderman of Albert Einstein College of Medicine in New York City is “overjoyed” by the report. “This smashes the paradigm that lower is better,” he says.

Researchers on both sides of the salt debate can point to hundreds of papers—sometimes the same ones—to make their case (*Science*,

14 August 1998, p. 898). Many studies have examined the effect of sodium on blood pressure, a predictor of cardiovascular health. But the Centers for Disease Control and Prevention asked the IOM panel to examine specifically whether cutting back on salt lowers the risk of “cardiovascular events,” such as a stroke or a heart attack.

The report confirms that Americans’ average daily sodium intake—3400 mg, most of it through processed food such as bread, pizzas, and restaurant meals—is too high, says Theodore Kotchen, an endocrinologist at the Medical College of Wisconsin in Milwaukee. “The science is fairly clear that reducing salt to a modest level—about 2300 mg a day—“is beneficial not just for blood pressure but for cardiovascular events,” he says.

The row centers on what happens at lower sodium consumption levels. While many researchers believe that the heart and blood vessels further benefit, some recent studies have suggested that a lower salt intake might actually increase risk. The curve describing the relation between sodium consumption and risk could resemble a J or a U, says Brian Strom of the University of Pennsylvania, who led the IOM study group.

The evidence is far from clear-cut. It mainly comes from studies on patients treated for serious diseases, which means there is a danger of confusing cause and effect, Antman warns. For instance, patients with heart failure or metastatic cancer might have a low sodium intake simply because they eat less. “This is not solid evidence,” he says.

Still, there are mechanisms by which a low-sodium diet might do harm. Lowering sodium activates the sympathetic nervous system and the renin-angiotensin hormone system, which helps regulate the body’s water

Shaking up the evidence. Reducing salt intake to less than 2300 mg a day may not reduce health risks, a panel says.

balance, and it increases triglyceride levels in the blood. “All of these have been shown to increase the risk of cardiovascular disease,” Alderman says. And because some sodium-rich products are laden with other minerals as well, a low-sodium diet could lead to a lower intake of other nutrients, like potassium, known to protect against cardiovascular disease. If so, drastically reducing salt might erode health because other micronutrients fall below a critical threshold, Strom says.

That is one reason why looking at salt alone is the wrong approach, says Connie Weaver, a nutritionist at Purdue University in West Lafayette, Indiana. She notes that in people who follow other dietary recommendations, salt intake appears to be less important. Encouraging people to eat better is a more promising strategy for protecting health, Weaver says. “Sodium,” she says, “is just easier to measure and make public policy around.”

Additional studies could help sort out these issues—but they are hampered by methodological problems. To measure sodium intake, some scientists use food questionnaires, a notoriously inaccurate method because people have trouble remembering what they ate. Urine samples provide a more reliable measure, but they’re unpopular with study participants.

Ideally, scientists would like to do prospective studies, in which people are randomly assigned to different sodium intakes and followed long-term. That would require participants to radically change their eating habits and would take thousands of participants and many years of follow-up to establish an effect. “While it makes sense on an intellectual level to ask for this, it is an impractical suggestion,” Antman says.

Experts crafting new U.S. dietary guidelines to come out in 2015 certainly won’t have such trials to lean on. Instead, Antman says, they should rely on the blood pressure reduction seen below 2300 mg. “We know hypertension is not a good thing to have,” he says, “and we have guidelines to treat it.”

Alderman is not convinced. What ultimately counts is whether people have a higher likelihood of suffering a stroke or a heart attack, he argues. “If you are going to ask people to change something, you need to have at least a clue that there will be a benefit.”

—KAI KUPFERSCHMIDT

CREDIT: BARBARA HELGASON/ISTOCKPHOTO

ANIMAL COGNITION

Can Animals Envision the Future? Scientists Spar Over New Data

Imagine you're a researcher who is about to conduct an elegant experiment. You muster all your scientific experience, the experiment goes beautifully, and years later your phone rings off the hook with congratulations from colleagues because you just won the Nobel Prize. You fly to Stockholm, mount the dais, and there's the king of Sweden waiting to—

Whoa, you're really getting ahead of yourself! Of course, we humans do this all the time. We use our past memories to construct visions of the future that may never come true. Researchers call it mental time travel (MTT), a term coined in the 1990s by psychologists Thomas Suddendorf of the University of Queensland in St. Lucia, Australia, and Michael Corballis of the University of Auckland in New Zealand. The pair maintained that MTT, which often involves conscious thought, is unique to humans. They laid down strict criteria for proving that animals can also do it, which they insisted had never been met even by the cleverest nonhuman species.

Ever since, other researchers have performed hundreds of experiments to test whether animals can do MTT, which is key to planning ahead. Now, Corballis, inspired by new studies of rat brains, has changed his mind, and cognition experts are taking notice. Last month, in *Trends in Cognitive Sciences*, the still-friendly but now divided duo debated each other. Corballis argued that the studies suggest that rats not only replayed maze paths they had run, but also mentally explored paths they never actually went down—a form of MTT. Suddendorf stoutly defended the pair's original position.

Researchers working with animals that appear to plan ahead have argued that some nonhuman species must do MTT, even if there's no way to demonstrate consciousness in these animals. They cite, for example, jays that cache food to eat later, or an irritable chimpanzee named Santino who seems to hide stones to be later hurled at zoo visitors.

Suddendorf insists that these observations

are not proof of MTT. "Animals share some" of the components of MTT, he says, including the ability to map external physical spaces in the brain and to store knowledge about the world around them. "However, mental time travel is more than that, allowing us to flexibly imagine any scenario, plot our future, and prepare for eventualities."

Corballis doesn't claim that animals have humanlike imaginations. But he says that the behavioral tests for MTT that he and Suddendorf concocted—including using novel problems and single trials so that animals couldn't pass by learning—may be too strict. The new work with rats, Corballis says, "provides deeper information" about what animals might be capable of.

The recent studies involved recording neural impulses from the hippocampus of rats running mazes. The hippocampus is key to memory storage in both humans and animals, "and is critical to mental time travel in humans," Corballis says. The breakthrough work was led by neuroscientist A. David Redish of the University of Minnesota, Twin Cities, and published in 2007 (*Science*, 9 November 2007, p. 900). Redish found that so-called place cells in the hippocampus, neurons which correspond to a mental map of the maze and fire in a sequence that mimics the maze's geography, fire ahead of the rat's actual location, and even along paths the animal may

only consider and not actually follow.

Bolstering that finding, researchers showed in *Science* last year that as rats learn a maze, their hippocampal neurons replay previous and possible future paths, even when they are resting outside the maze (15 June 2012, p. 1454). And a paper in *Science* last month showed that bats use place cells to map out the 3D spaces they fly through (19 April, p. 367).

The growing body of work shows that "mental time travel has ancient origins," Corballis argues, although he concedes that it is much more complex in humans. And Redish says that despite "clear differences" between rats and humans, he is "completely convinced at this point that animals can mentally time travel."

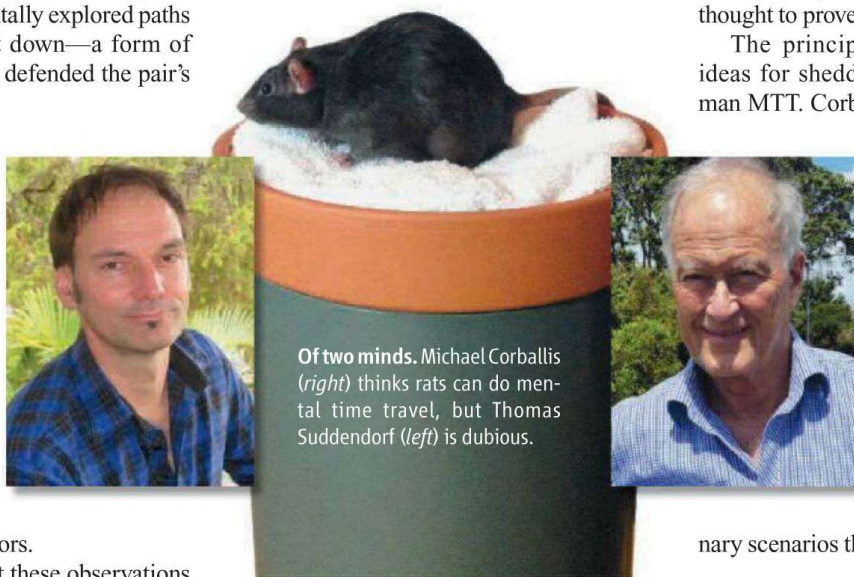
Suddendorf counters that rats are a few components shy of the full MTT package. For example, they lack subjective consciousness and so-called recursive thought: the ability to combine images and experiences to construct an infinite number of future situations as fanciful as winning a Nobel Prize, or traveling to planets long ago and far away.

Some animal behavior experts say that nonhuman MTT is for real—but for different reasons than Corballis has articulated. Behavioral studies of chimps and birds that cache food for later (*Science*, 23 February 2007, p. 1074) "are much more compelling" than the rat findings, says primatologist Mathias Osvath of Lund University, who has studied Santino, the stone-throwing chimp at Furuvik Zoo in Gävle, Sweden. Such studies demonstrate planning, and thus MTT, in nonhuman species, he says. He contends that it's not necessary to show conscious or imaginary thought to prove that animals engage in MTT.

The principal debaters have different ideas for shedding further light on nonhuman MTT. Corballis favors neurophysiological studies like the rat hippocampus work, whereas

Suddendorf thinks that if animals do engage in MTT, "more careful" behavioral tests should be able to detect it. He and Corballis do agree on one key point: Only with the evolution of language were animals able to start sharing their tales of mental time travel with each other, resulting in imaginary scenarios that could actually come true.

—MICHAEL BALTER



Of two minds. Michael Corballis (right) thinks rats can do mental time travel, but Thomas Suddendorf (left) is dubious.

Long Noncoding RNAs May Alter Chromosome's 3D Structure

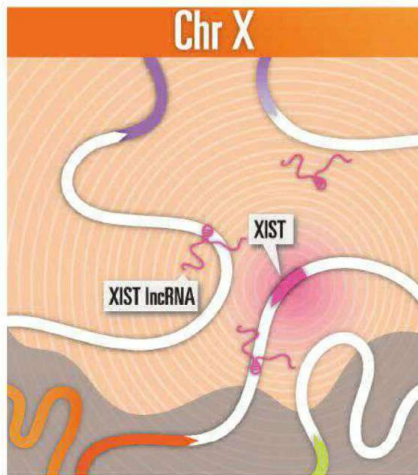
Our 21,000 protein-coding genes aren't the only readable units in our genome. At last count, another 13,000 "genes" specify mysterious molecules called long noncoding RNAs (lncRNAs), and when the final tallies are in, they may outnumber protein-coding genes. But what are these RNAs good for? Some researchers have suggested that they represent "noise": DNA randomly converted to RNA that serves no purpose. Others propose that they may be as pivotal as proteins in guiding cellular processes. To find out, Jesse Engreitz, a graduate student working with Mitchell Guttman and Eric Lander at the Broad Institute in Cambridge, Massachusetts, has taken a close look at one of the first noncoding RNAs discovered, XIST, which was identified 20 years ago as a silencer that shuts down one of the X chromosomes in females to ensure the proper amount of gene activity.

Engreitz has found that XIST operates by interacting with loops of nearby chromosome. "It seems to be creating a three-dimensional organization, bringing together regions of the genome in a way that we had assumed proteins were doing," says Emmanouil Dermitzakis, a genomicist from the University of Geneva in Switzerland. This finding supports a role for lncRNAs in regulating chromosomal activity by influencing the shape of chromatin, the protein complex that swaddles DNA. "It gives us a model of how other lncRNAs might be active," Dermitzakis adds.

Discovered in the early 1990s, XIST—along with the few other long noncoding RNAs known at the time—was considered an anomaly. XIST's gene is located on the X chromosome. As it converts to RNA, XIST spreads over the X chromosome, silencing genes. After 2 decades of study, researchers

still do not know how this spreading occurs or how XIST recognizes which parts of the X to inactivate.

When Engreitz arrived in Guttman's lab 2 years ago, the team was developing a way to see where along the genome a particular lncRNA would bind. Together, they came up with a method that uses RNA probes complementary to the lncRNA to target, bind, and precipitate out parts of the genome. When Engreitz tested this approach with XIST, he found that it bound to the X chromosome, but not where he expected. "It seems to bind everywhere," he said.



Reaching out. To silence genes on the X chromosome, XIST produces lncRNAs, which diffuse to nearby loops of DNA.

The scientists wondered if chromatin's 3D arrangement might come into play. Other researchers had used a method called Hi-C to build a 3D map of the twists and turns of the X chromosome. When Engreitz and his colleagues compared this map to their map of where XIST begins to bind, they saw a tight correlation with twists and turns close to where the XIST gene was located. "Where XIST goes first are the [DNA] sites that contact the XIST [gene]," he reported at the meeting.

In one experiment, Engreitz and his colleagues moved XIST 50 million bases down the X chromosome and put that altered X chromosome in mice embryonic stem cells. XIST interacted with a new set of DNA loops nearby. And when they put the XIST gene on a different chromosome, they saw a similar shift in binding. The results "clearly showed that physical proximity and interaction with the chromatin, and not sequence specificity, is important for spreading X-inactivation," says Piero Carninci from the RIKEN Center for Life Science Technologies in Yokohama, Japan. "This is quite impressive."

Other studies have shown that as XIST inactivation proceeds, XIST seems to reel in

the outer loops of the X chromosome, possibly by recruiting proteins that alter chromatin's conformation. "It's possible that lncRNAs represent a new type of gene regulator," says Rory Johnson, a genomicist at the Centre for Genomic Regulation in Barcelona, Spain.

Preliminary results with other lncRNAs suggest that they, too, may work like XIST, Engreitz reported. Other researchers point out that lncRNAs are abundant and may work in many different ways. "We just don't know," Johnson says.

—E. P.

In Latino Genomes, A Rich Source of History

Carlos Bustamante wants to know how much of human history is etched in our genomes. A population geneticist at Stanford University in Palo Alto, California, he and his colleagues have closely examined the DNA of Latinos in South Florida and traced their African, European, and South American ancestries. The team uncovered a stunning record of exploration, conquest, and slavery over the past 5 centuries, they reported at the meeting. "The results are a clear example of how genetics can trace back recent population history," says David Comas, a geneticist at Pompeu Fabra University in Barcelona, Spain.

Bustamante hopes to reach back even deeper into time. "We'd like to take this approach to far more ancient events," even thousands of years in the past, that involve the intermixing of different groups of people where written records are sparse. He also sees a practical benefit: Understanding the genetic history of individuals will help a clinician assess whether they share rare variants of genes that correlate with disease.

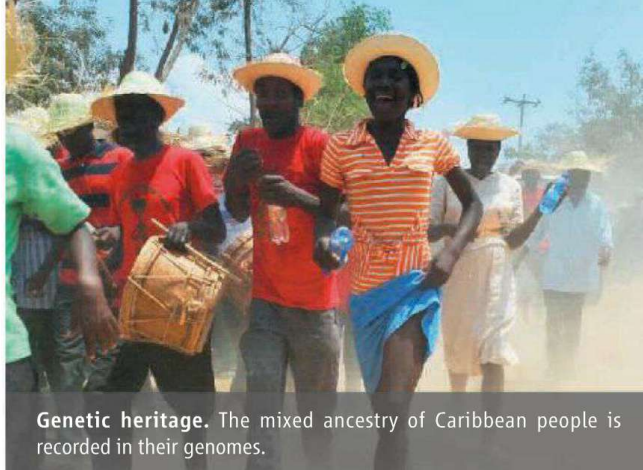
For the current study, geneticist Eden Martin of the University of Miami in Florida collected and analyzed DNA from Floridians who said that they had grandparents from three islands—Cuba, Puerto Rico, or Hispaniola—as well as those with families from Honduras and Colombia. They also looked at genetic data from three native South American tribes. The aim was to study the structure of their chromosomes.

When a couple has children, they donate entire chromosomes to the offspring, a complete set from each parent. But with each generation, chromosome pairs swap pieces

of DNA, mixing up the ancestry of the chromosome more and more. Segment lengths reflect how recently they were incorporated into the genome; shorter ones are older, as they have had more time to recombine with unrelated DNA. The result is a mosaic of DNA segments with different histories. Such admixtures complicate parsing out the genetic genealogy.

Bustamante, Stanford population geneticist Andres Moreno-Estrada, and their colleagues came up with a way of looking at subsets of DNA segments identified as coming from just one ancestral group. They did this by comparing the Latinos' DNA to that collected from only Europeans, Native Americans, or Africans.

The comparisons suggest that the Caribbean's original settlers came from the Ori-



Genetic heritage. The mixed ancestry of Caribbean people is recorded in their genomes.

noco Basin in South America. The European contribution came from Spain and Portugal, and the low diversity of this DNA indicates that very few individuals contributed to the gene pool; they were the early European explorers of this hemisphere. The researchers subdivided African DNA segments into long and short groups, esti-

mating that the short ones represented older DNA introduced about 450 years ago. These were most similar to DNA from present-day Senegal and Gambia. The longer, younger segments came from West Africa, signaling a second wave of slave trade from what is now Cameroon and Congo, according to Moreno-Estrada.

The results bode well for learning more about history from our genomes. "They are just getting started," says Goncalo Abecasis, a computational geneticist at the University of Michigan, Ann Arbor. "As we collect information on more genomes and develop better statistical algorithms, we will be about to reconstruct the history of individuals and populations with increasing detail."

—ELIZABETH PENNISI

U.S. SCIENCE POLICY

NSF Says No to Legislator Seeking Reviewer Comments

The National Science Foundation (NSF) last week rebuffed a request from the chairman of the House of Representatives science committee for reviewer comments that helped the agency decide to fund five projects in the social sciences.

The agency's staunch defense of confidentiality as an essential part of its vaunted peer-review system constitutes round two in what is becoming a protracted battle with Republicans in Congress over the agency's grants-making process. Each side says it's waiting for the other to take the next step.

On 25 April, Representative Lamar Smith (R-TX) wrote to acting NSF Director Cora Marrett asking for "access to the scientific/technical reviews and the Program Officers Review Analysis" for the five funded projects (*Science*, 17 May, p. 801). Smith and other Republican legislators have said that the grants raise serious questions about how NSF chooses from among 40,000 research proposals each year. Marrett replied on 15 May.

"I am disappointed the NSF declined to provide Congress with additional information that would show why they are spending taxpayer dollars on specific research grants," Smith said in a statement issued after receiving NSF's letter, a copy of which was shared with *Science*. According to a committee aide, NSF officials last month told the committee to describe the desired information in writing and that the agency would provide it.

NSF's response, says the aide, "is at variance with that conversation." NSF officials dispute that account and say no such promise was made.

The agency's 1.5-page letter explained how NSF's process works and asserted that the agency followed it in awarding the five grants. It described the importance of confidentiality and noted that any breach of that principle—either by identifying reviewers or by sharing their comments with a third party—could undermine the process and violate federal privacy laws.

After taking that hard line, Marrett extended an olive branch. "I hope that there may be another way to help the committee understand how NSF makes the decision to approve and fund grants short of the approach outlined in your letter," Marrett wrote, offering to set up a briefing for committee members.

On 20 May, Marrett told a meeting of advisers to NSF's social sciences directorate that the agency has "a responsibility to listen to lawmakers and try to decipher what's taking place and figure out how to respond." After the meeting, she briefly spoke to *Science*, saying she assumes that the next step is "a response from the chairman. We haven't heard from him yet."

"I hope that there may be another way ... short of the approach outlined in your letter."

—CORA MARRETT,
ACTING NSF DIRECTOR

The science committee aide described NSF's response as a bump on the road to obtaining the reviewer comments. "I called them right after we got the letter and said, 'Let's get together to work this out.' So the ball is in their court," says the aide, who spoke to *Science* after being promised anonymity. "We are trying to find a way to get the information we want."

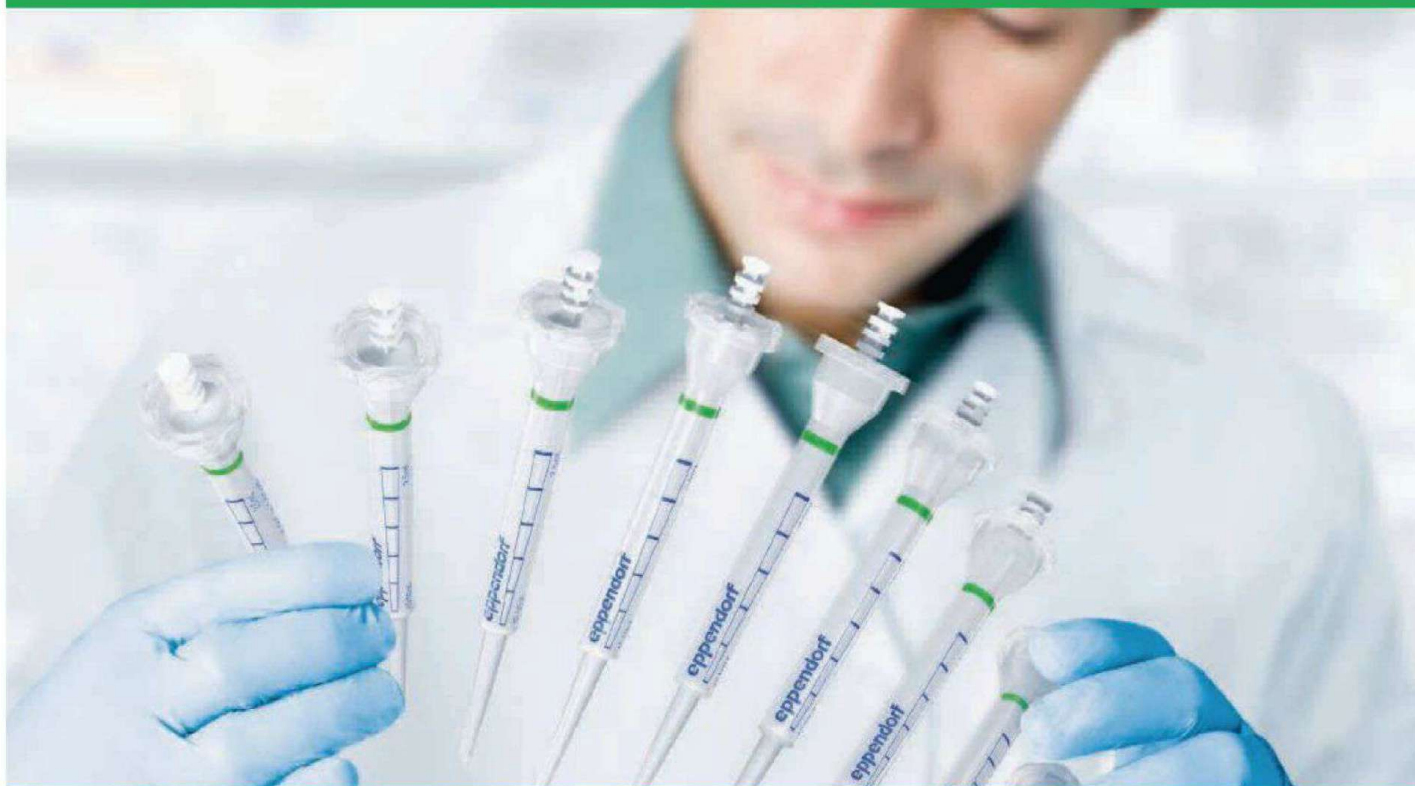
The committee is not interested in the identities of the reviewers, the aide notes. Rather, it wants to know why these five specific projects warrant NSF support. "I have worked with redacted statements before," the aide says. "It's such a small percentage of the overall content."

In March, Congress blocked NSF from funding any political science research this year unless it served to promote national security or economic development. Last month, Smith drafted a bill that, in effect, would apply such a test to NSF's entire research portfolio. Both the letter and the proposed legislation have inflamed the scientific community, which has urged Smith to rescind his request for information and abandon any legislation aimed at altering NSF's peer-review system.

—JEFFREY MERVIS

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MATHEMATICS

Two Proofs Spark a Prime Week for Number Theory

Last week, in a remarkable game of intercontinental “can you top this,” number theorists announced breakthroughs on two of the oldest unsolved problems in their subject. Although neither problem is close to being fully solved, the most dramatic progress in decades galvanized mathematical chat boards and provided instant fodder for seminars around the world.

The results concerned prime numbers, integers divisible only by themselves and 1. Primes are the building blocks of number theory, much as elements are the building blocks of chemistry. One of the problems, the “twin prime conjecture,” states that twin primes—those that occur two numbers apart, like 3 and 5 or 1091 and 1093—keep occurring ad infinitum as numbers get larger and larger. The other, Goldbach’s conjecture, makes two assertions: that every even number greater than 2 is the sum of two primes, and that every odd number greater than 5 is the sum of three primes. Mathematicians think that both conjectures are almost certainly true, but in more than a century and a half of trying, no one has come close to proving either one.

Then, on 13 May, Harald Helfgott, a 35-year-old Peruvian-born mathematician at the École Normale Supérieure in Paris, posted a preprint with a proof of the ternary (three-prime) part of Goldbach’s conjecture. On the same day, in a lecture at Harvard University, Yitang (Tom) Zhang of the University of New Hampshire in Durham sketched a proof that there are infinitely many prime numbers separated by less than 70 million. They may not be twin primes, but at least they are 70-millionth cousins.

“This is one of the great results in the history of analytic number theory,” says Andrew Granville of the University of Montreal in Canada of Zhang’s work. “It’s just extraordinary. I would never have expected it in my lifetime.”

Panning for twin primes

Both Zhang and Helfgott based their proofs on long-established plans of attack. For Zhang, the trick was to treat primes on the number line like gold nuggets in a stream and pan for them using the mathematical equivalent of a long sluice box. He developed a systematic way of telling when two primes were in the “box” at the same time. Then, Zhang used his new prime-detecting machinery to show that a “sluice box” 70 million units long is long enough to turn up an unending supply of slightly consanguineous pairs.

The number 70 million isn’t special and will surely shrink in future proofs, says John Friedlander, a number theorist at the University of Toronto in Canada. “The important thing is that there *is* such a number,” Friedlander says. “Forty million wouldn’t be any more exciting, and 140 million wouldn’t be any less.”

Zhang submitted his paper to the *Annals of Mathematics* on 17 April. On 9 May, after two referees gave it glowing reports, journal editor Nicholas Katz asked Zhang if he could issue a public announcement. Harvard invited him to give the mathematical world its first glimpse of his proof in a 1-hour seminar held on 13 May, and by the end of the week more speaking invitations were flooding in. It’s virtually unheard of, Granville says, for such a major result to come from a midcareer mathematician not previously noted for research in this area of number theory. “It’s beautifully written, just stunning, really masterful work,” he says.

Circle game

Unlike Zhang, Helfgott gave other mathematicians plenty of warning that he was closing in on a proof of the ternary Goldbach conjecture. “[T]he problem is now mortally wounded,” he announced on a popular mathematics blog last year.

Helfgott used a technique familiar to number theorists, called the Hardy-Littlewood-Vinogradov circle method. The main tool is Fourier analysis, the same method used in physics and engineering to decompose a periodic signal into a spectrum of its individual frequencies.

In the circle method, mathematicians construct a periodic function whose spectrum consists of all prime numbers. In 1923, G. H. Hardy and John Littlewood showed that

cubing this function and taking its spectrum would yield all the numbers that can be represented as the sum of three primes. Thus, to prove the ternary Goldbach conjecture, it’s enough to show that no odd-numbered frequencies are missing from the roster. In 1937, Ivan Vinogradov proved that all frequencies above some threshold were present, and other

mathematicians estimated that the limit was about 10^{1300} . As Hardy and Littlewood had done, Helfgott split the computation of the Fourier coefficients into two parts, called “major arcs” and “minor arcs.” The major arcs are like holes in a Swiss cheese, while the minor arcs comprise the rest. But Helfgott’s was more like a Muenster cheese: He was able to make the holes much smaller than Vinogradov had. Helfgott brought the limit down to 10^{30} ; then his colleague David Platt numerically verified the conjecture for every number below it, a

task that consumed about 40,000 hours of donated computer time.

Unfortunately, Granville says, for technical reasons Helfgott’s approach has “zero chance” of yielding a proof of the first, two-prime half of Goldbach’s statement, known as the “strong Goldbach conjecture.” He is slightly more optimistic that Zhang’s new theorem might provide clues to the twin prime conjecture.

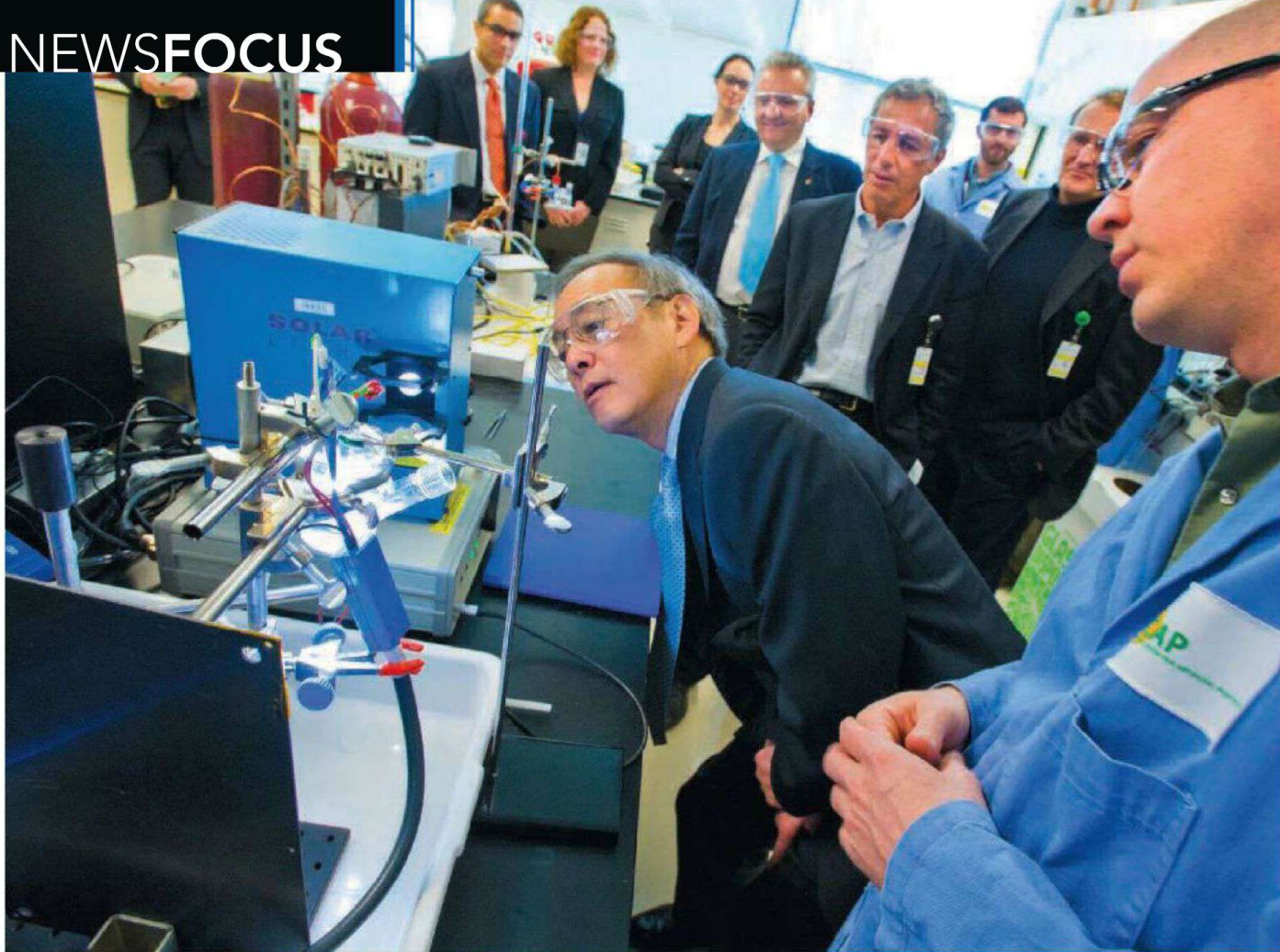
After generations of glacial progress on both problems, however, number theorists like Roger Heath-Brown of the University of Oxford in the United Kingdom were delighted to see signs of a spring thaw. “Despite hundreds of years of history, number theory is still moving on,” Heath-Brown says. “That’s what I find most exciting about these two theorems.”

—DANA MACKENZIE

Dana Mackenzie is a writer in Santa Cruz, California.



Stunner. Yitang Zhang’s proof about relationships among primes took colleagues by surprise.



Hubs Aim to Reinvent DOE Research Culture

The U.S. Department of Energy has funded a series of energy innovation hubs to tackle big energy challenges from start to finish. But their future is uncertain

EIGHT NUCLEAR ENGINEERS SIT IN HIGH-backed leather chairs, their laptop computers perched on sleek wood consoles. The lights are so low that the corners of the room fade to black. Up front, a roomwide video screen shows a half-dozen other researchers from locations around the country. A cryptic conversation rattles on: “Rod, we have to be careful. Right now the Cobra to de Novo isn’t even using DTK.”

Welcome to the Consortium for Advanced Simulation of Light Water Reactors (CASL) at Oak Ridge National Laboratory in Tennessee, one of five “energy innovation hubs” launched by the Department of Energy (DOE) to tackle tough problems. The

researchers participating in the virtual meeting at CASL are scrolling through a vast list of tasks as they integrate different computer programs for simulating the inner workings of nuclear reactors so that utilities can run them more efficiently.

The brainchildren of Steven Chu, the Nobel Prize-winning physicist who stepped down last month as secretary of energy, the hubs are a bold experiment in how DOE does research. Modeled after the Manhattan Project that built the atomic bomb and the famed Bell Labs, the hubs aim to make the sprawling and notoriously bureaucratic agency more nimble and responsive. Each would focus on a single problem, assemble

“under one roof” all the scientists and engineers needed to tackle everything from basic research through technological development, and take a free-wheeling approach to hammer out a solution as fast as possible.

In 2009, as soon as he became energy secretary, Chu announced plans to create eight hubs. To date, DOE has launched five, funding each for \$122 million over 5 years with the possibility of a 5-year renewal. In 2010, in addition to CASL, it established hubs that focus on generating fuel from sunlight through artificial photosynthesis and reducing energy consumption in buildings. In recent months, DOE has launched hubs to create far better batteries and to head off shortages of materials. And DOE’s proposed budget for 2014 requests money for a sixth hub on electrical systems such as the power grid.

The problem-oriented approach draws plaudits from researchers and some policymakers in Washington, D.C. “The hub is exactly what DOE should be doing,” says Charles Dismukes, a physical chemist at Rutgers University, Busch Campus, in Piscataway, New Jersey. A Republican Senate staffer agrees: “I think you’re going to see an expansion of these, and the traditional

CREDIT: ROY KALTSCHMIDT

Check it out. Then–Energy Secretary Steven Chu visited the Joint Center for Artificial Photosynthesis in April 2012.

[DOE research] program may stagnate as a result.”

Before that can happen, however, the hubs will have to prove their worth at a time when budgets are likely to be tight. Evaluating them will not be easy, in part because they widely vary in the ways that they are organized and the types of problems that they are tackling. For example, only one of the five strives to be a physical center of activity; the rest are widely distributed collaborations. In fact, the hubs vary so much that it's difficult to say exactly what a hub is. And some see a risk that, now that Chu is gone, the hubs could be subsumed into the department's existing bureaucracy and their independence compromised.

Breaking down the stovepipes

To understand what a hub is supposed to be, it helps to understand how DOE is structured. Sometimes called the Department of Everything, DOE has a \$24.4 billion annual budget and comprises a dozen different offices and agencies, such as the National Nuclear Security Administration, which safeguards the United States' nuclear weapons, and the Office of Energy Efficiency and Renewable Energy (EERE), which develops clean energy technologies. An office may be divided into programs, such as the \$1.6 billion Basic Energy Sciences (BES) program within the \$4.7 billion Office of Science.

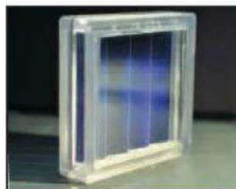
DOE has a reputation for being badly “stovepiped,” meaning that the various offices and programs poorly communicate with one another. For example, in theory the BES program should yield breakthroughs in basic research that the EERE program then develops through its applied research. In practice, “BES does its thing and then they throw it over the transom and hopefully it hits somebody at EERE in the head,” says William Madia, vice president at Stanford University for DOE's SLAC National Accelerator Laboratory in Menlo Park, California, a national lab mainly supported by BES.

The hubs aim to break down the barriers between basic and applied research. Chu shaped the concept by calling on his experience at Bell Labs, the research wing of the AT&T phone company that from the 1920s through the 1990s produced technologies ranging from the transistor to the laser. Chu did his Nobel Prize–winning work there,

HUBS CAPSULES

JOINT CENTER FOR ARTIFICIAL PHOTOSYNTHESIS (JCAP)

Locations	Founded	Goal	Prospects
California Institute of Technology in Pasadena and Lawrence Berkeley National Laboratory in California	2010	Produce affordable, durable, and efficient solar cell that generates hydrogen fuel from sunlight and water.	The most hublike hub, JCAP strives to keep all its research literally under its two roofs. To reach their goal, researchers must overcome numerous challenges in basic research.



CONSORTIUM FOR ADVANCED SIMULATION OF LIGHT WATER REACTORS (CASL)

Locations	Founded	Goal	Prospects
Oak Ridge National Laboratory, Tennessee	2010	Create detailed 3D simulations of existing reactors to enable utilities to improve efficiency.	CASL has already produced new insights into reactors. Success will require the Nuclear Regulatory Commission to approve the new simulations in licensing new fuel designs and fuel cycles.



ENERGY EFFICIENT BUILDINGS (EEB)

Locations	Founded	Goal	Prospects
Philadelphia, Pennsylvania Lead institution: Pennsylvania State University	2010	Improve energy efficiency of buildings in the Philadelphia metropolitan area by 20% by 2020.	As well as tackling technological problems, EEB researchers study the economic and regulatory issues affecting building efficiency. Some observers question whether that diverse approach is appropriate for a hub.



JOINT CENTER FOR ENERGY STORAGE RESEARCH (JCESR)

Locations	Founded	Goal	Prospects
Argonne National Laboratory, Illinois	2012	Develop within 5 years a battery that holds five times as much energy as a standard lithium ion battery at one-fifth the cost.	JCESR researchers say that they ought to be able to meet their crisply defined goal. But turning theory into reality in 5 years will be tough.



CRITICAL MATERIALS INSTITUTE (CMI)

Locations	Founded	Goal	Prospects
Ames National Laboratory, Iowa	2013	Head off shortages of key elements such as rare earth metals by finding replacements and better means of extraction and recycling.	CMI leaders say that they seek to become a standing resource for those in the field. Some observers question if that fits with the hubs' problem-oriented approach.



and he says the lab succeeded because it let scientists and engineers quickly hash things out for themselves.

"The managing scientists and engineers were the bosses," Chu says. "Unlike a 3-year grant where you write a proposal and it takes a year to get funded, at Bell Labs if I had an idea I'd go to my boss or my boss's boss and get a yes or no in days or weeks."

As at Bell Labs, a hub is supposed to bring together all the people and resources needed to perform everything from the basic research and development to demonstration and deployment of a prototype solution. "The distinctive signature of the hub is that it goes from basic research all the way through to deployment all under one roof," Madia says. "You can't find that kind of activity when you look at the DOE stovepipes."

The target areas for the hubs have been chosen by top DOE officials, including Chu himself. Teams then submitted proposals and competed for each hub, with the proposal detailing a team's approach to the problem.

The archetypal hub

No hub embodies the center-of-action concept used at Bell Labs more closely than the Joint Center for Artificial Photosynthesis (JCAP). Headquartered at the California Institute of Technology (Caltech) in Pasadena and funded by the BES program, JCAP (pronounced J-cap) aims to create a technology that could ease both the world's looming energy crunch and climate change caused by burning fossil fuels. "We want to have something that you can hold in your hand that will produce fuel from sunlight in a way that humans haven't done before—with no wires and no microbes," says Nathan Lewis, a chemist at Caltech and JCAP's founding director and chief scientist.

The basic idea of "solar fuels" isn't new. Plants store solar energy in sugars through photosynthesis, so a refinery that generates ethanol from corn creates fuel from sunlight. JCAP researchers aim for something more direct—using solar energy to split water and generate combustible hydrogen gas. That can be done in the lab by, for example, using a wafer of silicon plated with platinum on one side and iridium on the other. Using solar energy captured by the silicon, the iridium

catalyzes the breakup water into oxygen molecules, hydrogen ions, and electrons, while the platinum combines the electrons and ions into hydrogen molecules. Other approaches use different catalysts.

But none of those approaches is ready for the real world. For a solar-fuels industry to flourish, its core technology must be efficient, durable, and cheap. No existing technique nails all three requirements, says



Cyber center. Video conferencing links researchers at the widely dispersed Consortium for Advanced Simulation of Light Water Reactors.

Carl Koval, an electrochemist at Caltech and JCAP's director. The platinum and iridium scheme is efficient and robust, for example, but iridium is rare and expensive. JCAP's goal is a cheap, durable cell that is 10 times as efficient as photosynthesis.

As the hub concept stipulates, JCAP researchers grapple with every aspect of the problem, breaking it into eight distinct projects. Interconnected groups strive to identify new catalysts and light-capturing materials, make sure they'll work together, integrate them into structures sculpted on the nanometer-scale, and design the macroscopic cell. Some of JCAP's goals are wildly ambitious. Researchers plan to characterize a million catalysts per day using a modified inkjet printer to print tiny dollops of the materials on glass slides. That would be more catalysts in 1 day than have been characterized in history, Lewis says.

Perhaps most striking is the enthusiasm with which JCAP officials embrace the "one roof" concept. JCAP leaders say that their biggest organizational challenge is to prevent JCAP from becoming just another university research center. William Royea, a chemist and JCAP's assistant director for strategy and communications, characterizes such a center as "a confederation of research projects in which everybody goes off and

does whatever they want." JCAP leaders see physical proximity as the best means to avoid that loss of focus.

In particular, JCAP leaders want to prevent scientists from viewing the hub as just a source of funding for work that they're already doing in their own labs. To keep researchers focused on building the device, JCAP officials insist that they work in JCAP's cozy building. "If you're on JCAP, you're here every day. You're in the building," Lewis says. (JCAP actually has two buildings, with a northern branch at Lawrence Berkeley National Laboratory in California. However, its leaders note that Bell Labs also had multiple campuses.)

The flow of money also follows the Bell Labs' model. Funding goes not to individual principal investigators but to the eight project leaders who distribute it as they see fit.

Virtual centers

Although JCAP strives to be a center of activity, the other hubs are extended collaborations. For example,

CASL comprises 10 institutions including DOE's Idaho National Laboratory in Idaho Falls; the Electric Power Research Institute (EPRI) in Palo Alto, California; and Westinghouse Electric Company. Most researchers visit Oak Ridge only every other month. "I love living here," says Douglas Kothe, a nuclear engineer at Oak Ridge who leads CASL. "But a lot of people don't and a lot of people won't. So our one-roof model is to aggressively deploy virtual-collaboration technology."

That's fine, observers say, because the cost of moving people and equipment could easily consume all the money that the hub receives from DOE. The real issue, they say, is whether a hub tackles the right kind of problem—one big enough to require a hub's resources, but not so big that it can't be solved in 5 years. By all accounts, CASL has found just such a problem: simulating the inner workings of the nuclear reactors in service today.

Funded through DOE's \$720 million Office of Nuclear Energy, CASL is an attempt to play catch-up. The nuclear industry pioneered the use of simulations, Kothe says. But the United States has not broken ground for a new nuclear power plant since 1977, and as the nuclear industry stagnated, so did the simulations. "This field is a couple of decades

CREDIT: COURTESY OF CASL

behind” the automotive and aerospace industries in using simulations, Kothe says.

Still, such simulations are crucial for demonstrating to the Nuclear Regulatory Commission (NRC) that any changes to a reactor’s design or operations will be safe. The 104 U.S. reactors now run at 92% of capacity, up from less than 80% 20 years ago, says John Gaertner, a nuclear engineer and CASL member from EPRI. To improve efficiency and reduce costs, utilities would like to further increase power levels, the length of fuel cycles, and the extent to which fuel is depleted, or “burn-up.” Current simulations largely rely on data from reactor tests to make semiempirical predictions, says Daniel Ingersoll, a nuclear engineer with NuScale Power in Portland, Oregon, who does not work on CASL. That limits how much plant owners can change designs and operations, he says.

CASL aims to produce 3D simulations that more fully capture the underlying physics and chemistry, says Paul Turinsky, a nuclear engineer from North Carolina State University in Raleigh and CASL’s chief scientist. Current 2D simulations track the flow of cooling water around the fuel rods in the reactor’s core at hundreds of points; CASL’s simulations will track it at a billion points. Turinsky says that CASL’s simulations will also encompass the tricky interactions between the radiation emanating from the fuel rods, the flow and heating of the water around the rods, and the effects of the radiation on materials such as the rods’ metal cladding.

CASL is leading the other hubs in generating results, observers say. To ensure that their work is practical, CASL researchers are also analyzing operational challenges facing reactors. One is the buildup of deposits known as CRUD—for their historical name, Chalk River Unidentified Deposits—on the fuel rods, which can distort the rods’ power output. CASL researchers have already predicted a CRUD pattern that was later detected on rods. “We consider that a major advance,” says William Martin, a nuclear engineer and CASL member from the University of Michigan, Ann Arbor.

CASL researchers hope that industry will use their simulations to improve the design of fuel assemblies and fuel cycles. Developing the simulations is too costly for industry alone, says Zeses Karoutas, a nuclear engineer and CASL member with Westinghouse in Columbia. “Industry can’t put together the kind of funding that’s needed,” he says. “The only way to do it is to leverage the national labs and the universities.”

Soft focus

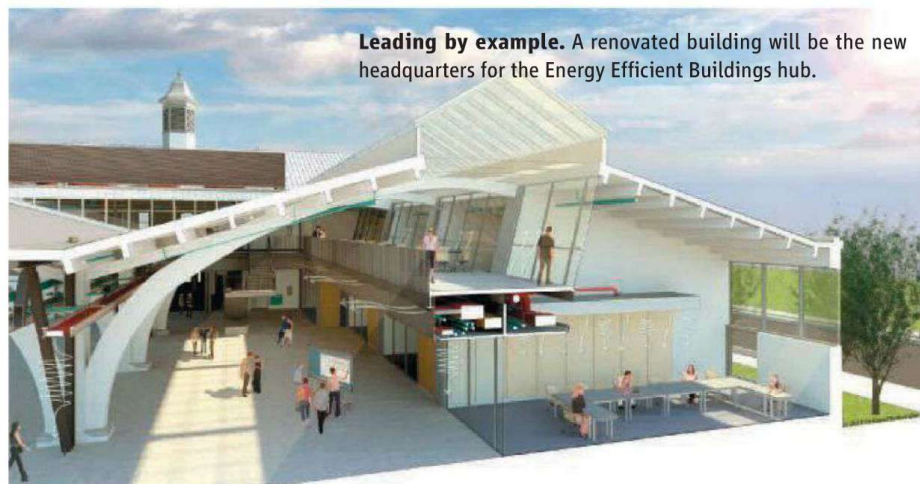
Some hubs are not focusing on a specific problem, an approach that has created some controversy. The Energy Efficient Buildings (EEB) hub in Philadelphia, Pennsylvania, which is mainly funded by EERE, aims to improve energy efficiency in area buildings by 20% by 2020. Buildings soak up 40% of the energy used in the United States, says James Freihaut, a fuel scientist at Pennsylvania State University (Penn State), University Park, and chief scientist for EEB. So if implemented nationwide, such an improvement would save massive amounts of energy.

Buildings have become only marginally more efficient in recent decades, even though the efficiency of subsystems such as heating, air conditioning, lighting, ventilation, and electrical power have improved by larger amounts, Freihaut says. That disparity arises because builders do not consider how the various subsystems will interact, he says. “We’re trying to transform the building industry as a whole into a system provider,” he says.

way membranes to draw moisture out of a building or building materials whose capacity to hold heat changes with temperature, energy use could drop by between 30% and 50%, Freihaut predicts.

However, improving building efficiency is not a single well-defined technological problem, but rather a raft of various technological, economic, and regulatory issues. For example, the Philadelphia City Council drew upon one of EEB’s studies for an ordinance that requires the owners of commercial buildings bigger than 4700 square meters to publicly report a building’s water and energy consumption. The law, passed in June 2012, is supposed to give tenants a clearer idea of their energy costs and owners an incentive to make buildings more efficient.

EEB’s work is focused less on developing new technologies and more on persuading builders to adopt ones that already exist. “We actually don’t want to be too much on the leading edge because building owners are naturally conservative,” says Laurie Actman, an engineer at Penn State and deputy director of EEB.



Leading by example. A renovated building will be the new headquarters for the Energy Efficient Buildings hub.

To do so, EEB researchers are developing computer modeling tools to predict how different combinations of specific subsystems will interact. The need for such tools became apparent when EEB researchers went to retrofit their current headquarters, an old building in Philadelphia’s Navy Yard, Freihaut says. Three different consulting firms gave entirely different assessments of what needed to be done, he says.

Using their own building, EEB researchers are also studying how many measurements of temperature, humidity, air flow, electricity use, and other factors are needed for a building to be “self-aware” enough to optimize its energy consumption. With foreseeable advances in materials, such as one-

All that work may be worthy, but some Washington insiders question whether it requires a \$122 million hub. In fact, multiple sources on Capitol Hill say that EEB’s future is uncertain. “We have serious concerns about the building hub,” says a staffer for the Democratic majority in the Senate. “If this doesn’t turn around in the next few months in terms of clarifying what we’re getting for our investment, you may see some action.”

The same issue of focus has come up with regard to one of the two latest hubs. One of them seems to neatly fit the hub model in having a crisply defined technological goal. In November, researchers at Argonne National Laboratory in Illinois won the competition for a hub to develop far better bat-

teries. The Joint Center for Energy Storage Research (JCESR) aims to produce within 5 years a battery that can hold five times as much energy as a standard lithium ion battery and can be made for one-fifth the cost.

In contrast, the Critical Materials Institute (CMI) at Ames Laboratory in Iowa, which launched in January, will tackle the complex question of how to ease shortages of vital elements such as the one that arose in 2009 when China threatened to stop exporting neodymium, europium, and other rare earth metals. CMI researchers will take a four-pronged approach, doing the basic materials science to find ways of replacing or reducing the use of key materials, exploring better ways to extract materials from ore, pursuing advanced recycling, and trying to predict shortages.

In doing so, however, CMI researchers seek to solve not a single technological challenge, but rather to meet a variety of challenges as they arise. "What we are seeking to develop is a resource—the SWAT team for materials shortages," says Alexander King, a materials scientist and director of Ames lab and CMI. That description raises some eyebrows in Washington. "The original notion of a laser focus on a specific problem that you'd know whether you'd solved it or not is totally unclear on that one," says one Republican House of Representatives staffer.

Life after Chu

It's too early to tell whether each hub will reach its technological goal. And the standard will vary. For example, for JCAP to claim success, researchers must simply come up with a practical prototype. And some say that's a long shot. "It will only work if the science is there, and it's not there yet," says Richard Eisenberg, a chemist at the University of Rochester in New York.

In contrast, CASL's success will depend on whether NRC approves its simulations for use in licensing applications. CASL's work so far is impressive, says Jennifer Uhle, deputy director for reactor safety programs in NRC's Office of Nuclear Reactor Regulation. However, approval requires "validating" the simulations' accuracy with reactor data, she says. Given the level of detail in the simulations, data for such validation may be hard to get right away, Uhle cautions. "You can't measure the flow [of

water in a reactor] at a billion points without disrupting the flow you're trying to measure," she says.

In spite of the uncertainties, the hubs appear to enjoy support in both houses of Congress. Compared with DOE's traditional research program, the hubs' problem-oriented approach makes it easier for legislators to see what they're getting for their money, says the Democratic Senate staffer. Still, the staffer cautions, to prove their worth the hubs have to produce results that couldn't be achieved otherwise.

The view from the Republican-controlled House is more circumspect. Anxious to protect DOE's basic research pro-



Hub honchos. From right, Douglas Kothe of CASL, Nathan Lewis of JCAP, Henry Foley of EEB, George Crabtree of JCESR, and Alexander King of CMI at a recent press event in Washington.

grams, some Republican legislators worry that the hubs may duplicate research within DOE and elsewhere. For example, the Republican House staffer says, the federal government already funds 39 initiatives on battery research, including 11 within DOE.

At the same time, House Republicans seem willing to give the hubs a chance. "If you're going to start these things, you should at least finish the first 5 years and look at the metrics and see how these things have succeeded," the staffer says.

Ironically, the biggest threat to the hubs could come from within DOE. The hubs are supposed to break with DOE's traditional way of structuring research. To allow researchers to react quickly to promising leads, the hubs are supposed to be managed with a "light federal touch," says Alexander Larzelere, DOE's program manager for CASL in Washington, D.C. To maintain that autonomy, Larzelere is DOE's only point of contact for CASL, and he says he must make a concerted effort to fend off other bureaucrats. "I spend most of my day trying to keep people from mucking with the hub," he says.

However, that streamlined arrangement means CASL answers to only DOE's Office of Nuclear Energy. So it runs counter to the idea that the hubs should span the stovepipes, says Stanford's Madia. In fact, people have begun to talk about the hubs as belonging to one or another office, he says, so that the batteries hub is an Office of Science hub and the materials hub is an EERE hub.

Such a mindset, Madia says, threatens to obscure a hub's mission to span the spectrum of research, development, demonstration, and deployment of a technology. "If it's going to work, you need a healthy amount of each of these four pieces," he says. "If you get it out of balance, you're just replicating what DOE is already doing."

In fact, some researchers argue that the independence of the hubs is undermined by DOE's failure to follow through on all aspects of the concept. The hubs were supposed to answer to a hubs board, and a hub "champion" within DOE would stick up for them personally, says Paul Hallacher, a political scientist and director of EEB. But those things never materialized, Hallacher says. "There is no DOE hubs program," he says. "There are merely five hubs lodged within different offices." But others argue that

such a board would only add another level of bureaucracy to the system.

With no concrete mechanism to guarantee that the hubs maintain just the right amount of autonomy, the fate of the hubs may well depend on how strongly they are embraced by Chu's successor. On 4 March, the Obama administration nominated Ernest Moniz, a nuclear physicist from the Massachusetts Institute of Technology in Cambridge who served as undersecretary in DOE from 1997 to 2001, to be the next energy secretary.

Hubs' proponents are optimistic that Moniz, who was unanimously confirmed last week by the U.S. Senate, will see the value of their work. He's certainly familiar with the concept, having chaired CASL's board of directors from the hub's inception until last year. Still, observers say, Moniz will have lots of other issues to attend to and may have initiatives of his own for which he'd rather push now that he is in office.

Even if Moniz does embrace the hubs' concept, his first task may be to define exactly what a hub is.

—ADRIAN CHO

CREDIT: COURTESY OF DOE



On thin ice. The wolves of Isle Royale are dwindling in number and face an uncertain future.

National Park. But some researchers argue that the study's value is so great and the current wolves so integral to the island that they warrant a "genetic rescue," by importing other wild wolves soon.

After discussions with geneticists, Peterson now leans toward this option as the "most beneficial, giving the best scientific return." But the fate of the wolves rests with the park service.

One for the books

The story is a staple of biology textbooks. Moose arrived on Isle Royale

in the early 1900s, probably by swimming the 24 kilometers from the Ontario shore. Their numbers exploded on the predator-free island, and their browsing took a toll on the boreal forest. Then in 1949, a breeding pair of wolves crossed the frozen stretch of lake and began preying on the moose. The two populations began their scientific careers in 1959, when wildlife biologist Durward Allen of Purdue University and graduate student L. David Mech recorded the first numbers—20 wolves and about 500 moose—in what has become the world's longest running predator-prey study and one of science's most famous data sets.

The study has borne out some of ecology's fundamental tenets. It provided the first indisputable evidence that wolves hunt old and infirm moose or calves while leaving healthy adults alone. It was one of the first studies to show top-down control of a terrestrial food chain, by connecting wolf predation to the health of trees as seen in tree rings (*Science*, 2 December 1994, p. 1555). "Trees grew better when wolves were high and moose were low," Peterson says.

Since the pioneer wolves padded across an ice bridge to the island, the tale of Isle Royale has testified to the power of random events. "The significance of rare, unpredictable events is enormous," Peterson says, and often only understood in the

long term. For example, in the 1980s the wolf population crashed from its peak of 50 to a mere dozen (see timeline, p. 920). Years later, researchers found out why, detecting signs of deadly

ECOLOGY

Are Isle Royale's Wolves Chasing Extinction?

Wolves in an iconic predator-prey study are not producing pups, leaving scientists to confront a genetic rescue—or the project's demise

Since his graduate student days in 1971, Rolf Peterson has spent a few nights of every summer camped within earshot of wolf packs on Isle Royale, Michigan, in the northwestern corner of Lake Superior. It may be as early as the end of June that he hears the sound he's waiting for: high-pitched yips that stand out from the long, sonorous howls of adult wolves. The calls herald a new generation of pups joining the island's storied wolf population. For 40 years without fail, Peterson and his colleagues have heard those yips.

Until last summer. For the first time, the wildlife ecologist at Michigan Technological University in Houghton heard no yips in 2012, and failed to spot pups during the official aerial population count in January 2013. The lack of reproduction means that 2012 will likely go down as the beginning of the end of Isle Royale's wolves. The January count put them at their lowest number ever—only eight closely related individuals, down one from last year. The Michigan Tech team is on the island now and will listen intently for pups this summer, but Peterson

believes that inbreeding's pernicious effects have doomed the wolves. "It's over," he says. "It's just a matter of watching it wind down."

The surprise may be that the inbred population on the 544-square-kilometer isle has hung on this long, more than 6 decades after it was established by two or three curious wolves from nearby Canada. One school of thought has maintained that this chance natural experiment should run its course even if that means extinction, followed by a transfusion of fresh wolves to control the moose population on the rugged island, which is a U.S.

Online

sciencemag.org

S Podcast interview with author Christine Mlot (http://scim.ag/pod_6135).



Science island. Isolated Isle Royale is home to the world's longest-running predator-prey study.



canine parvovirus in the wolves (*Science*, 27 August 1993, p. 1115). Even later, Peterson learned how parvo was likely brought to the island: A Minnesota veterinarian reported treating a parvo-infected dog that illegally visited the island on 4 July 1981. By 1990, the virus apparently burned itself out and disappeared from the island.

It's not just the wolves that have yielded scientific bounty: The moose, too, have been a research bonanza. Researchers and volunteers have packed out bones from close to 5000 moose carcasses, resulting in the world's largest collection. Together with bones from about 50 wolves, these remains have yielded insights on topics including forest health and moose arthritis.

For example, while teaching a dissection class, Peterson noticed that the club-sized lower leg bone of moose from the mainland seemed larger than what he was used to in Isle Royale specimens. His team has since shown that the size difference is significant and in a paper in press in the journal *Alces* will report that male Isle Royale moose have smaller antlers, too. In just a century on the island, moose have downsized to become among the smallest in the world. Thus, they are a classic case of another key biological insight: Large mammals shrink on islands, as did the miniature hippos and elephants that lived on Mediterranean islands during the Pleistocene. "On islands, big things get smaller," says evolutionary biologist Shripad Tuljapurkar of Stanford University in Palo Alto, California. The Isle Royale findings, he says, "provide good evidence that the island rule applies."

Inbreeding's insidious effects

Now that the wolf population has sunk to an all-time low, with just two remnant packs



Fresh stock. The immigrant "old gray guy" (center) reinvigorated Isle Royale's inbred wolves in the late 1990s.

instead of the historical three or four, this natural experiment in population biology and island biogeography is turning into a grim portrait of inbreeding depression: the reduced biological fitness that plagues tiny populations.

At least half of the eight wolves counted in January are female, according to DNA tests of scat, but the researchers detected less courting and mating than usual during their winter survey. The lack of reproduction may be because wild wolves typically avoid mating with close kin—and there are no other options on Isle Royale. Or, the wolves are mating but infertile because of genetic anomalies from inbreeding, or their offspring may not be viable.

In any case, it's clear that the wolves are racking up more and more physical abnormalities, probably from inbreeding. A 2009 paper in *Biological Conservation* reported that 58% of examined wolves had congenital spinal deformities, compared with only 1% of wolves in other populations. The abnormalities appear widespread—they turned up in all 12 wolves necropsied recently—but apparently are

not crippling, Peterson says.

Other observations bolster the argument that inbreeding is eroding the population's fitness. Researchers have noted wolves with one opaque, perhaps blind, eye. Also, an apparently healthy female died in her den in 2009 after delivering one pup, with her remaining seven pups dying in utero. That

had never been documented before in a wild wolf and may have ultimately stemmed from inbreeding, Peterson says.

As dark as the outlook looks for the wolves, Isle Royale also offers compelling evidence of how to revive an inbred population. In 1997, in another chance event unbeknownst at the time to researchers, a large male wolf from Ontario crossed the ice bridge to the island. In 1998, the scientists spotted a striking animal—which project co-leader and Michigan Tech population biologist John Vucetich later nicknamed "old gray guy"—that became

whiter as he aged, something not seen before in Isle Royale wolves. But his provenance didn't become clear until after 2007, when Jennifer Adams, then a postdoctoral researcher at Michigan Tech and now at the University of Idaho in Moscow, extracted DNA from stores of frozen wolf blood, bone, and scat and analyzed it for key microsatellite markers to identify individual wolves.

The wolf DNA markers were rigged to fluoresce when exposed to a laser and recorded by software as distinctive peaks. As Adams ran the results for a marker on the



Inbred. Abnormalities such as an opaque eye and stillbirths plague Isle Royale wolves.

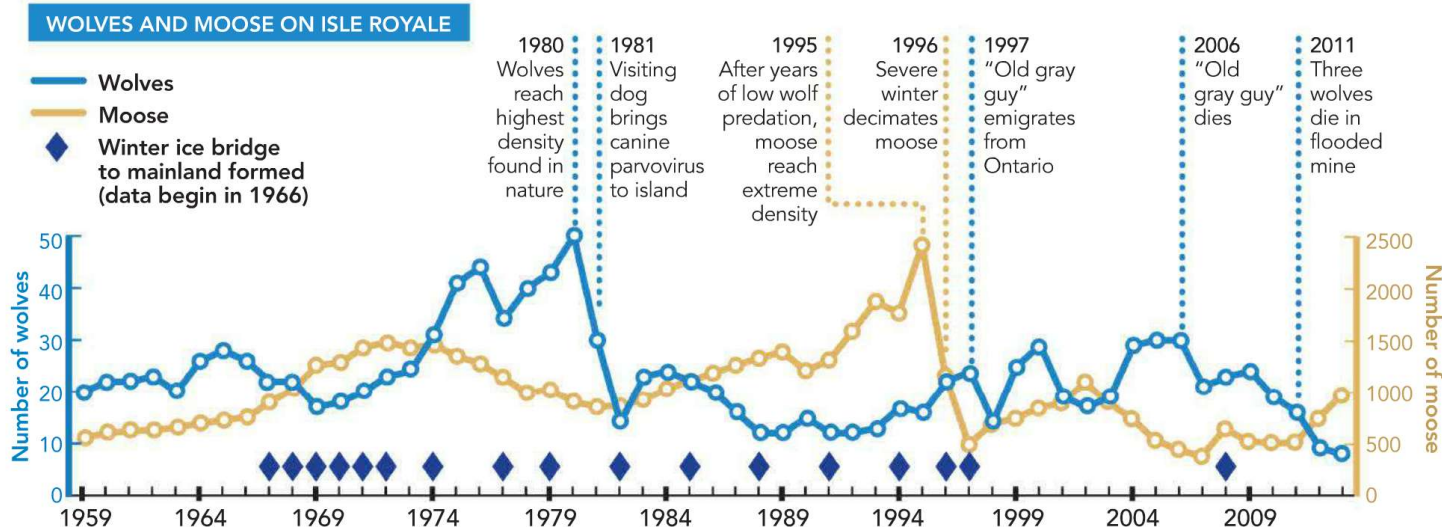


PHOTO CREDITS (TOP TO BOTTOM): JOHN VUCETICH/MICHIGAN TECHNOLOGICAL UNIVERSITY; ROLF O. PETERSON/MICHIGAN TECHNOLOGICAL UNIVERSITY (2); GRAPH: ADAPTED FROM J. A. VUCETICH AND R. O. PETERSON 2013 ECOLOGICAL STUDIES OF WOLVES ON ISLE ROYALE, ANNUAL REPORT 2012-2013, MICHIGAN TECHNOLOGICAL UNIVERSITY, Houghton, MI. USA DATA COLLECTED AND ANALYZED BY D. ALLEN, D. LICH (NFS) AND R. GITZEN/UNIVERSITY OF MISSOURI

Y chromosome to identify males, she found a common peak representing one characteristic Y chromosome. As she ran the data for later years, a second peak suddenly appeared, representing a different Y chromosome marker. By the time she got to the 2003 samples, the first marker type had disappeared. When she constructed a family tree based on the genetic markers, the second type markers all traced back to one wolf.

Peterson and Vucetich went back to their field notes on the scat sample that had yielded the new Y chromosome: They had observed the old gray guy defecate at the site of a winter moose kill and had taken a sample. The new Y chromosome marker was independent corroboration of what researchers had noted in the field—the gray guy had taken over one pack as the new breeder.

Breed he did. After mating with a female and later with his own daughter, the old gray guy sired 34 offspring and genetically took over Isle Royale before his death in 2006. After a single generation, 56% of the genes in the population were his. His Y chromosome is now the only one present in the population. “Such a dramatic selective event influencing the whole genome has never been documented in a wild population before, as far as I know,” says Arizona State University, Tempe, population geneticist Philip Hedrick, one of Adam’s co-authors on a 2011 report on the phenomenon in the *Proceedings of the Royal Society B*.

Old gray guy’s appearance in 1997 “saved the population for another 10 to 15 years,” Peterson says. As wolf numbers grew, moose numbers fell, reinvigorating vegetation. “He had an impact on the entire forest of Isle Royale,” Peterson says. The immigrant also opened up a new research vein. University of California, Los Angeles, evolutionary biologist Robert Wayne and colleagues are now sequencing the genome of Isle Royale wolves before and after the old gray guy’s arrival, to see how inbreeding affects genome architecture and to compare mutations with those of outbred wolves.

Old gray guy’s new genes weren’t a cure-all, however. With the wolves dwindling once again, the population clearly could use another infusion of new blood.

But the odds are slim that more Canadian wolves will find their way onto Isle Royale. Temperatures in the region are rising with the changing climate, and the formation of winter ice bridges has become a once-a-decade event, instead of once every two to three winters as in the 1960s. Recent hot summers also seemed to be affecting the cold-adapted moose, which were down to about 385 in

2007. With wolves on the wane, however, moose are now waxing, from about 750 in 2012 to 975 at the start of 2013.

What to do?

Last winter, the researchers and the park service solicited input from geneticists about the wolves’ decline. But opinion on the path forward is divided. Citing successful genetic rescues of other populations, such as the Florida panther (*Science*, 24 September 2010, p. 1641), Hedrick and others advocate a similar operation on Isle Royale. Their argument rests on the wolves’ importance to the ecosystem and their influential scientific legacy. “It’s so incredibly rare to have such a long-term data set,” says conservation geneticist Lisette Waits of the University of Idaho. Advocates also note that the current wolves have cultural knowledge of the island. Although other populations hunt moose, Isle Royale is distinctive in that a single predator basically depends on a single prey. The population may not be genetically different from Ontario’s *Canis lupus*, but “I don’t think you should say they are just like every other population,” Hedrick says.

Vucetich is open to a genetic rescue and the lessons it could offer to other populations on the brink. Yet, he says there’s still a chance—a very small one—that the wolf population can come back on its own. “Isle Royale wolves are in the business of surprising us,” he says. Mech, now with the U.S. Geological Survey, notes that the recent moose decline could have delayed wolf breeding. With last winter’s moose calf boom, pups could still arrive in the next few years.

The park service generally takes a hands-off approach in wilderness areas, but it’s giving Isle Royale special scrutiny. The agency has assembled a team of national policy experts, local wildlife managers, and others to review the plight of the wolves in the context of how to treat the species in national parks as a whole. Options under consideration are to do nothing, bring in new wolves as soon as possible, or reintroduce wolves once the existing population dies off, which could happen in the next 5 years. The researchers are adamant that wolves are integral to Isle Royale’s ecosystem. “As long as there are moose on Isle Royale, there should be wolves,” Vucetich says.

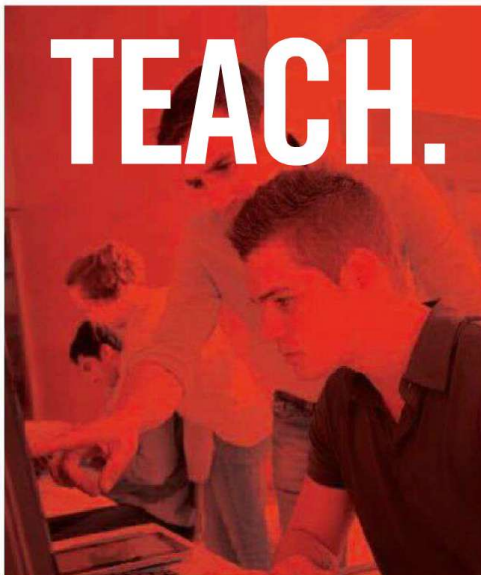
Climate change is also a big part of the discussion, says Isle Royale park Superintendent Phyllis Green, who expects the team to recommend a course of action by fall. At that point, a public consultation will kick in, although anyone can weigh in now by sending comments to ISRO_Wildlife@nps.gov.

The park service decision will determine the fate of only a few animals on a remote island. But given the increasing number of isolated wildlife populations, Green says, this is “a national decision that will affect national policy.”



Moose multitude. With wolves scarce, moose numbers are up, which will add to the thousands of bones Peterson (above) and colleagues have collected over the years.

In the meantime, researchers and park visitors alike remain on watch. Earlier this month, volunteers on the project were lucky enough to spot three wolves hanging out on a frozen bay. The threesome probably was one of the packs, Peterson says—which means that the female isn’t curled up in a den with weeks-old pups. So the lucky sighting may bode ill for the wolves. Whether these wolves blink out, revive, or face another unpredictable game-changing event, observers on and off the island will be watching. —CHRISTINE MLOT
Christine Mlot is a Wisconsin-based writer.



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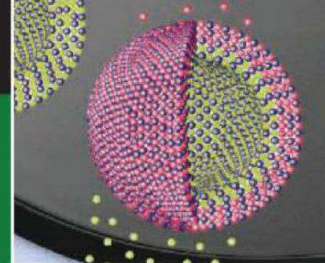
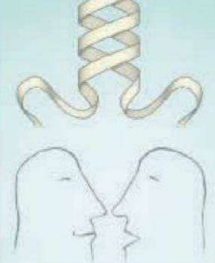
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LETTERS

edited by Jennifer Sills

Shark Mislabeling Threatens Biodiversity

AS COMMERCIAL FISHERIES STRUGGLE TO APPLY REGULATORY AND LEGAL MECHANISMS THAT depend on reliable species-specific data (1), the shark industry faces an even greater obstacle to transparency: Sellers change product names to overcome consumer resistance. For instance, South Africa sells shark meat (shortfin mako shark) mislabeled as “ocean fillets” or “skomoro” and in doing so threatens a vulnerable species (2). Conversely, the European Union (3) requires listing the species name on shark products to avoid fraud and to help conserve certain species (4).

The situation is even worse in many developing countries [e.g., Mozambique, Costa Rica, India, Sri Lanka, and Borneo (5)], where shark meat is commonly sold without proper identification. In Brazilian supermarkets, elasmobranchs (members of a fish subclass distinguished by the lack of swim bladders) are sold as “cação,” a popular name for any small shark species or pup. Consumers do not understand that cação refers to any elasmobranch, regardless of its size or species. This intentional mislabeling compromises efforts to lessen shark consumption or to promote consumption of nonthreatened species.



For sale. Shark meat in a Brazilian market.

Only a few isolated initiatives have been attempted to force supermarkets to better inform customers of which shark species they are consuming. For example, on 1 July 2011, Instituto Justiça Ambiental (the Environmental Justice Institute) filed a public civil action against the Walmart and Carrefour supermarket chains requesting that they sell shark meat with appropriate scientific species identification (6). This is a critical step for shark conservation everywhere, and especially in Brazil, where 18 shark species are threatened, overexploited, or under threat of overexploitation (7).

Meanwhile, another five oceanic shark species and manta rays were recently added to the list of animals whose trade requires permits as described by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). With CITES oversight, the international trade of these species can only take place if the meat is shown to be obtained legally and sustainably (8).

Proper labeling and identification can be done by trained individuals monitoring elasmobranch landings from artisanal to industrial fisheries or in supermarkets with modern genetic identification techniques (4, 9, 10). With this action, the general public would be able to make educated decisions about whether or not to consume shark meat. This matter is fundamental to marine conservation and to maintaining sustainable and transparent seafood consumption.

HUGO BORNATOWSKI,¹* RAUL RENNO BRAGA,¹
JEAN RICARDO SIMÕES VITULE²

¹Universidade Federal do Paraná, 19020, Brazil. ²Laboratório de Ecologia e Conservação, DEA, Universidade Federal do Paraná, 19020, Brazil.

*Corresponding author. E-mail: anequim.bio@gmail.com

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Pollination Decline
in Context

THE REPORT BY L. A. GARIBALDI *ET AL.* (“WILD pollinators enhance fruit set of crops regardless of honey bee abundance,” 29 March, p. 1608; published online 28 February 2013) demonstrates that wild pollinators enhance production of many crop species. However, it is premature and possibly mistaken to relate this result directly to food production (“The global plight of pollinators,” J. M. Tylianakis, *Perspectives*, 29 March, p. 1532; published online 28 February 2013). Pollination is but one of many, often conflicting, factors that affect the production of animal-pollinated crops. Justifying conservation of pollinators (and pollinator habitat) on the basis of food production remains a spurious argument if other factors are not considered.

From a farmer’s perspective, management to enhance pollinators makes little sense if that same action has high opportunity costs.

Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

Californian almond farmers continue to intensify production despite declining honey bees because pollinator service costs are insignificant compared to the increasing returns from intensified production (1). Indian coffee farmers intensify production despite recognized costs to pollination because returns on coffee increases as a result of such action (1, 2). Globally, the rate of increase in crop production does not appear to differ between crops that do and do not depend on pollinators (3, 4), and in any case there is no doubt that intensification has increased production of all crops. Thus, although it is difficult to disagree that “biodiversity has a direct measurable value for food production,” the more important issue is whether management to secure biodiversity-related benefits is more rewarding for crop production than management less favorable to biodiversity. It is this measure

that will (or will not) persuade farmers to promote pollinators within agricultural systems.

We agree that many pollinators face serious threats. Along with other threatened species, their conservation is warranted. Yet pinning conservation arguments to food security is questionable if benefits to food production and farmer revenues compared to other management systems cannot be established. For the sake of farmers, policy-makers, and our own credibility as conservation scientists, let us consider the implications of pollinator decline in the context of the entire agricultural management system.

JABOURY GHAZOU

Ecosystem Management, Department of Environmental Systems Science, ETH Zurich, 8092 Zurich, Switzerland. E-mail: jaboury.ghazoul@env.ethz.ch

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Response

OBVIOUSLY NOBODY WOULD ARGUE THAT pollination is the only factor affecting food production, and of course other factors can limit the importance of pollination for any particular crop (1). The purpose of studies like that of Garibaldi *et al.*, and others from single-crop systems (2), is to determine whether diverse pollinator assemblages improve production all other things being equal. Garibaldi *et al.* clearly demonstrated that for 41 crop systems, diverse native pollinators do increase crop production, and Ghazoul presents no evidence to the contrary. Intensification of production can drive

CORRECTIONS AND CLARIFICATIONS

Reports: “A localized Wnt signal orients asymmetric stem cell division in vitro” by S. J. Habib *et al.* (22 March, p. 1445). In the legend to Fig. 4D, the last line should have stated: “Red bar: H3K27me3 stain in the bead-distal cell; yellow bar: H3K27me3 stain in the bead-proximal cell; blue bar: no H3K27me3 stain in any cell.” The HTML and PDF versions online have been corrected.

Reports: “Conduction of ultracold fermions through a mesoscopic channel,” by J.-P. Brantut *et al.* (31 August 2012, p. 1069; published online 2 August 2012). Equation 1 was incorrect. The correct version is below. The HTML and PDF versions online have been corrected.

$$\frac{d}{dt}\Delta N = -\frac{G}{C}\Delta N$$

Reports: “Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery” by M. Pal-Bhadra *et al.* (30 January 2004, p. 669). The author Madhusudana Rao Chikka was mistakenly listed by his first and middle names.

TECHNICAL COMMENT ABSTRACTS

Comment on “ApoE-Directed Therapeutics Rapidly Clear β -Amyloid and Reverse Deficits in AD Mouse Models”

Nicholas F. Fitz, Andrea A. Cronican, Iliya Lefterov, Radosveta Koldamova

Cramer *et al.* (Reports, 23 March 2012, p. 1503; published online 9 February 2012) demonstrated in a mouse model for Alzheimer’s disease (AD) that treatment of APP/PS1 Δ E9 mice with bexarotene decreased A β pathology and ameliorated memory deficits. We confirm the reversal of memory deficits in APP/PS1 Δ E9 mice expressing human APOE3 or APOE4 to the levels of their nontransgenic controls and the significant decrease of interstitial fluid A β , but not the effects on amyloid deposition.

Full text at <http://dx.doi.org/10.1126/science.1235809>

Comment on “ApoE-Directed Therapeutics Rapidly Clear β -Amyloid and Reverse Deficits in AD Mouse Models”

Ashleigh R. Price, Guilian Xu, Zoe B. Siemienski, Lisa A. Smithson, David R. Borchelt, Todd E. Golde, Kevin M. Felsenstein

Cramer *et al.* (Reports, 23 March 2012, p. 1503; published online 9 February 2012) demonstrates short-term bexarotene treatment clearing preexisting β -amyloid deposits from the brains of APP/PS1 Δ E9 mice with low amyloid burden, providing a rationale for repurposing this anticancer agent as an Alzheimer’s disease (AD) therapeutic.

Using a nearly identical treatment regimen, we were unable to detect any evidence of drug efficacy despite demonstration of target engagement.

Full text at <http://dx.doi.org/10.1126/science.1234089>

Comment on “ApoE-Directed Therapeutics Rapidly Clear β -Amyloid and Reverse Deficits in AD Mouse Models”

Ina Tesseur, Adrian C. Lo, Anouk Roberfroid, Sofie Dietvorst, Bianca Van Broeck, Marianne Borgers, Harrie Gijzen, Diederik Moechars, Marc Mercken, John Kemp, Rudi D’Hooge, Bart De Strooper

Cramer *et al.* (Reports, 23 March 2012, p. 1503; published online 9 February 2012) tested bexarotene as a potential β -amyloid-lowering drug for Alzheimer’s disease (AD). We were not able to reproduce the described effects in several animal models. Drug formulation appears very critical. Our data call for extreme caution when considering this compound for use in AD patients.

Full text at <http://dx.doi.org/10.1126/science.1233937>

Comment on “ApoE-Directed Therapeutics Rapidly Clear β -Amyloid and Reverse Deficits in AD Mouse Models”

Karthikeyan Veeraraghavalu, Can Zhang, Sean Miller, Jasmin K. Hefendehl, Tharinda W. Rajapaksha, Jason Ulrich, Mathias Jucker, David M. Holtzman, Rudolph E. Tanzi, Robert Vassar, Sangram S. Sisodia

Cramer *et al.* (Reports, 23 March 2012, p. 1503; published online 9 February 2012) reported that bexarotene rapidly reduces β -amyloid (A β) levels and plaque burden in two mouse models of A β deposition in Alzheimer’s disease (AD). We now report that, although bexarotene reduces soluble A β 40 levels in one of the mouse models, the drug has no impact on plaque burden in three strains that exhibit A β amyloidosis.

Full text at <http://dx.doi.org/10.1126/science.1235505>

Response to Comments on “ApoE-Directed Therapeutics Rapidly Clear β -Amyloid and Reverse Deficits in AD Mouse Models”

Gary E. Landreth, Paige E. Cramer, Mitchell M. Lakner, John R. Cirrito, Daniel W. Wesson, Kurt R. Brunden, Donald A. Wilson

The data reported in the Technical Comments by Fitz *et al.*, Price *et al.*, Tesseur *et al.*, and Veeraraghavalu *et al.* replicate and validate our central conclusion that bexarotene stimulates the clearance of soluble β -amyloid peptides and results in the reversal of behavioral deficits in mouse models of Alzheimer’s disease (AD). The basis of the inability to reproduce the drug-stimulated microglial-mediated reduction in plaque burden is unexplained. However, we concluded that plaque burden is functionally unrelated to improved cognition and memory elicited by bexarotene.

Full text at <http://dx.doi.org/10.1126/science.1234114>

both declines in biodiversity and increases in production—or even alter the importance of biodiversity for ecosystem processes (3). However, intensifying production to overcome declining biodiversity is not a sustainable way forward in a changing environment. The dust bowl in the Great Plains of the United States and increased salinization in Australia are sobering reminders of the long-term impacts of intensification on both the environment and production.

Ghazoul argues that “pollinator service costs [in Californian almonds] are insignificant compared to the increasing returns from intensified production.” Yet, in an entirely pollination-dependent crop like almond, this only remains true as long as honey bees can be purchased in large numbers at low cost. The suite of pests and diseases historically and currently threatening honey bees (4) makes them a precarious future foundation for entire industries. In times when honey bee abundance is low, wild pollinators (when available) have been shown to fill the gap in pollination (5).

Conservation of bee-friendly habitat in Germany was recently shown to generate

150% increases in yields of cherry by wild bees, whereas farmers had assumed that honey bees were the main cherry pollinators (6). Therefore, intensification and reliance on single species may well mask the costs of declining biodiversity in the short term, but they will not necessarily maximize crop production or its sustainability in the long term.

The key to Ghazoul’s argument is that “management to enhance pollinators makes little sense if that same action has high opportunity costs,” yet he provides no evidence to suggest that such opportunity costs are, in fact, generally high. The importance of opportunity costs to conservation on private land has been discussed for decades, and initiatives such as the EU Agri-environment schemes (7) were developed precisely to alleviate these costs to landowners. Even in developing countries, where no such schemes exist, there is growing evidence that wildlife-friendly farming approaches need not come at a cost to yield (8). Ecosystem services may even make threatened-species conservation actions by landowners economically beneficial (9).

Therefore, the important challenge will be to find local and landscape approaches to

maintain pollinator and other species diversity in agricultural landscapes (10), without generating large opportunity costs for landowners (11). Of course it won’t be easy, but this will be one of the most important scientific, social, and political challenges of the near future.

JASON M. TYLIANAKIS

School of Biological Sciences, University of Canterbury, Christchurch 8140, New Zealand. E-mail: jason.tylianakis@canterbury.ac.nz

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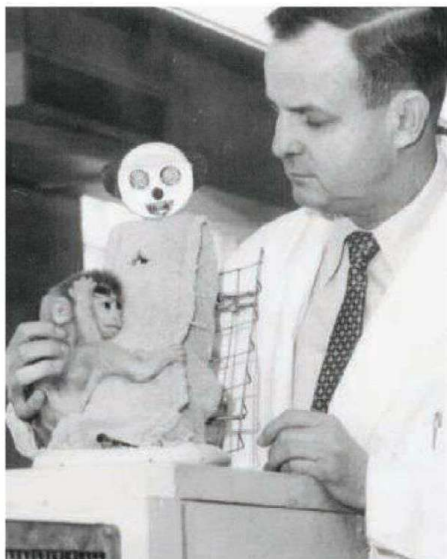
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PSYCHOLOGY

Mother, Love

Ben Harris

In Steven Spielberg's 2001 film *A.I.: Artificial Intelligence*, a young couple adopts a robot child (David) that has the capacity for emotions as well as rationation. This requires his human mother to activate an "imprinting protocol" that binds David to her as if his love were hard-wired, with her as its focus. Thanks to his programming interacting with his mother's love, the robot child becomes emotionally connected and human.



Superior monkey mother? Harry Harlow with an infant rhesus monkey and a cloth surrogate mother.

To Marga Vicedo, the imprinting protocol of *A.I.* is a futuristic expression of a theory of human development that has become influential in psychology, related disciplines, and popular culture. The theory posits that a mother's love for her infant is instinctual. A corollary is that such love is essential to the child's development. More specifically, empathic, motherly love must be expressed during an early, critical period in an infant's life. If successful, the child's emotions will develop normally, creating a healthy adult personality. Without the proper maternal love, however, the infant will be stunted and become a dysfunctional individual.

As the terms "critical period" and

"instinctual" suggest, this theory of attachment is modeled on Konrad Lorenz's ethological theory of animal behavior. Like the goslings that imprinted on Lorenz and followed him around, human babies are believed to develop an emotional attachment to their mothers if exposed to their love at the right moment. The creator and popularizer of modern attachment theory was British psychiatrist John Bowlby, who drew on Lorenz for scientific authority. Together they promoted a theory of human development and an analysis of family life and social organization in the post-World War II era.

In *The Nature and Nurture of Love*, Vicedo shows how Bowlby and his allies biologized motherhood and made the bond between mother and infant the key to creating emotionally healthy adults and solid citizens. A historian of science at the University of Toronto, Vicedo accepts no component of attachment theory as empirically revealed, natural truth. Rather, she historicizes its various features, assumptions, and modes of expression. In doing so, she reveals their implicit beliefs and normative prescriptions. Mothers, the theory explains, should love their babies naturally—without qualification or hesitation. They must not work outside the home or use daycare. Bowlby warned that "[t]o deprive a small child of his mother's companionship is as bad as depriving him of vitamins."

Vicedo's history centers on the Cold War era, when social commentators and the public debated the effects of changing gender roles in post-World War II families. Scientists, in turn, studied the effects of family disruption: children living in orphanages or being separated from parents due to the war. To Bowlby and Lorenz, such research on attachment and separation would help safeguard family integrity, which they linked to social stability and the struggle against communism.

Initially, Bowlby's evidence came from psychoanalytic studies, known for their anecdotal methodology and small sample sizes. What made his theory convincing was its later incorporation of experimental stud-

ies of animals—revealing laws of development across species, including humans. Of the animal researchers, Harry Harlow was the most influential, a sober-looking professor in a lab coat who dared to study "the nature of love." In his primate laboratory at the University of Wisconsin, Harlow raised infant rhesus monkeys on surrogate mothers that dispensed milk and had bodies made of either wire mesh or terrycloth.

The result that most pleased the attachment theorists was the social and sexual immaturity of Harlow's surrogate-raised monkeys. More dramatically, females raised on surrogates were the worst possible mothers to their own infants. Clearly, Bowlby said, early contact with a real mother is necessary for emotional development.

What Harlow actually showed, however, was that monkeys' contact with peers is the key to emotional development, with mother acting as a go-between. That discovery was, along with other anti-attachment findings, ignored by Bowlby and Lorenz.

As the reader learns, animal research was not the only discipline misrepresented by the radical instinctivists. Anna Freud, for example, complained that Bowlby excised the subjective, psychological essence of psychoanalysis in his fervor to biologize the infant-mother bond. In her chronicles of such disagreements between Bowlby and his critics, Vicedo's analysis of scientific evidence is thorough enough to be used in a course on research methodology.

As a historian of science, however, she is after bigger game. She asks how a scientific theory can endure when its evidence and logic are persuasively refuted by experts. Her answer is that Bowlby's attachment theory brought the authority of biology to the seemingly less rigorous field of developmental psychology. It also borrowed from enough scientific and social-scientific specialties to outflank critics who only knew one discipline. And compared to their opponents, Bowlby and Lorenz presented a united front that persisted for decades—while others moved to new research questions.

More than the story of a controversy in developmental psychology, *The Nature and Nurture of Love* is a compelling interrogation of a popular scientific theory, its creators, and its critics. Put on the witness stand, instinctual attachment theory does not acquit itself well.

The Nature and Nurture of Love
From Imprinting to Attachment
in Cold War America

by Marga Vicedo

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The reviewer is at the Department of Psychology, University of New Hampshire, Durham, NH 03824, USA. E-mail: bh5@unh.edu

PUBLIC HEALTH

Economic Rewards to Motivate Blood Donations

Nicola Lacetera,^{1*†} Mario Macis,^{2,3*} Robert Slonim^{4,3*}

The position and guidelines of the World Health Organization (WHO) and several national blood collection agencies for nearly 40 years have been based on the view that offering economic incentives to blood donors is detrimental to the quantity and safety of the blood supply (1). The guidelines suggest that blood should be obtained from unpaid volunteers only (2). However, whether economic incentives positively or negatively affect blood donations (and other prosocial activities) has remained the subject of debate since the positions were established (2–8).

Evidence consistent with the WHO position came originally from uncontrolled studies using nonrandom samples and, subsequently, from surveys and laboratory studies indicating that economic incentives can “crowd out” (decrease) intrinsic motivations to donate and can attract “worse” donors (9). This evidence arguably affected policies, such as bans on compensation for blood and organ donations in many countries.

Surveys allow for a variety of hypothetical manipulations on large samples, and laboratory experiments parallel laboratory health research methods by enabling researchers to carefully control the setting, randomize the assignment of treatment, and identify causal effects. Because compensation is illegal in many countries and observing blood donations is often costly (as only a small share of subjects invited to donate actually do so), surveys and laboratory studies retain an important role for addressing many questions (10). Yet it is unusual for health policy to rely only on such evidence. Complementary, randomized field trials are the norm and are recommended before policies are affected (11). With a few early exceptions based on small, nonrepresentative samples (12), field trial evidence on how economic incentives affect blood dona-



tions has been absent. But field-based evidence from large, representative samples has recently emerged. The results are clear and, on important questions, opposite to the uncontrolled studies, surveys, and laboratory evidence preceding them.

An Overview of the Evidence

Several fundamental problems make evidence from early studies on incentives for blood donations unreliable (9). Samples were often small or nonrandom, and controls for potentially confounding factors (such as the prevalence of first-time donors, the location of donations, and the use of prisoners) were not distributed equally between incentivized and non-incentivized subjects. We thus focus on studies relying on large, representative samples of existing or potential donors that control for confounding factors to better identify and isolate causal effects (table S1).

Surveys and framed experiments across several countries find that respondents generally state aversion to receiving money for donating blood (13–18). Attitudes are less negative, and sometimes positive, when rewards have less clear economic connotation, such as receiving free medical testing (e.g., a cholesterol test) (13–16). Women indicate more aversion to economic rewards (17, 18), and subjects more responsive to incentives report behaviors (e.g., drug use) that lead to a higher risk for transfusion-transmissible infections (e.g., hepatitis), which make them ineligible to donate (13, 14).

More recent research from published and working papers uses field-based methods with larger, representative samples, as well as clinical trial-like experiments with

Field-based evidence suggests that guidelines against economic rewards to motivate blood donors should be reconsidered.

minimal manipulation of the environment. Observational studies that control for confounding factors have examined 14 incentive items ranging from small coupons to a paid day off work. All were found to increase blood donations (19, 20). For example, items such as T-shirts and coupons led to 16% more donations at American Red Cross blood drives (20), and a 1-day paid leave was associated with 40% extra annual donations in Italy (19).

Three field experiments examining five different economic items offered to thousands of subjects in the United States and Switzerland examine nondonors (21) and existing donors (22, 23) among subjects who had been offered rewards never before (21, 22) or irregularly (about 35% of the time) (23). Similar to observational data, and again in contrast to the survey and framed studies, the first-order findings are large, positive effects of economic rewards. For instance, a 5 Swiss franc (~\$5.35) lottery ticket offer increased donations by 5 percentage points over a baseline of 42% (22), and a \$10 gift card offer increased U.S. donations by 7 percentage points over a 13% baseline (23).

Overall, 18 of the 19 distinct incentive items offered in observational and field experimental studies increased blood donations, and the effects were larger for items of higher monetary value (20, 23); only one reward offer, a free cholesterol test, had no effect (21, 22). When data were available (for 15 of the items), no effect on blood safety was detected (20, 22). Finally, although temporary rewards might affect long-term motivations, no post-intervention effects on donations were found, including any negative effects deriving from potential motivation loss (23).

Two additional important results are that incentives had spatial and short-term temporal effects on donations (23), which indicated that rewards can successfully address temporary shortages, and that no field study reports any gender differences on blood donations in response to reward offers.

There are many potential reasons for the differences between the results from the field and those from surveys and the laboratory. Subjects responding to hypothetical

¹University of Toronto and University of Toronto—Mississauga, Ontario L5L 1C6, Canada. ²Johns Hopkins University, Baltimore, MD 21202, USA. ³Institute for the Study of Labor (IZA), Bonn 53113, Germany. ⁴University of Sydney, Sydney, New South Wales 2006, Australia.

*All authors contributed equally to this article. †Corresponding author: nicola.lacetera@utoronto.ca

questions about socially desirable activities, like donating blood, may focus on seeing themselves in a positive perspective (24) and, thus, respond that they donate solely to help others; these same people may value a reward when actually offered one. Similar behavior may occur if subjects feel that researchers judge their actions and so wish to show that they donate for socially desirable reasons and not for rewards. In natural settings, there is no parallel to this perceived researcher scrutiny, thus subjects may be less concerned with loss in reputation (25, 26).

Implications for Policy and Future Research

These studies inform, yet limit, policy implications. First, because rewards were only offered one time or occasionally in all of the studies, we cannot infer the effect of offering rewards all the time. Nonetheless, the success of one-time or sporadic rewards is important because rewards can be offered at a specific time of greatest need, as shortages often occur at predictable times (e.g., winter). Further studies can determine if, and for how long, continued use of incentives may increase blood supply.

Second, the existing trials examine offers that are material items (e.g., T-shirts, lottery tickets, gift cards) rather than cash because cash is not allowed. No study observing actual donations has tested whether offering material items will work better than cash.

Third, items offered are framed as gifts or rewards rather than “getting paid.” The early debate on whether incentives undermine motivation to donate blood assumed that the incentives would be perceived as payment (3), rather than as gifts. Future research can address the importance of this difference in framing. In the meantime, the success of incentives not framed as a payment is strongly supported by the existing studies.

Fourth, rewards are not provided for making a blood donation, but rather for showing up to donate, which removes the incentive for people to provide false information so that they qualify to donate and consequently obtain the rewards. This practice may be critical for blood safety when incentives are offered.

Fifth, the evidence discussed so far comes from wealthy countries. However, shortages are more severe in resource-constrained economies because of inefficient blood collection systems that use emergency-replacement donations for specific recipients rather than anonymous, undirected donations (1). Only one field trial has examined economic incentive offers for undirected blood donations in a middle-income country, Argentina, where emergency dona-

tions are the norm (27). Consistent with the higher-income country results, supermarket vouchers of AR\$60 (~\$11.50) and AR\$100 (~\$19.20) increased undirected donations to 0.5 and 1.1%, respectively, from a baseline of no undirected donations in the no-reward condition and had no significant effects on blood safety compared with emergency donations. However, two other items with economic value tested in the study, a T-shirt and an AR\$20 (~\$4) supermarket voucher, had no effects, which highlights caution with extrapolating results to contexts with different institutions and norms.

Perhaps the key concern with blood donations in developing countries is safety, because testing blood is relatively more costly. More evidence is needed, but three insights from existing studies and current practice are noteworthy: (i) items offered in Argentina and elsewhere did not affect safety; (ii) offering rewards for presenting, rather than donating, should diminish donors falsifying information; and (iii) encouraging state-of-the-art blood testing can further allay safety concerns (28).

Finally, although we focused on studies of the effects of economic rewards, other mechanisms should be investigated. For instance, symbolic rewards and social recognition have enhanced donations among some groups, but not all (27, 29). Empathy and emergency appeals have increased donations among first-time donors in the United States after 9/11, whereas T-shirt offers had no effect on this group (30). The impact of a blood donor registry paralleling bone marrow and kidney registries is also worth exploring (31).

Conclusion

In light of the recent evidence, it is time to re-examine policy guidelines for increasing and smoothing blood supply, including whether incentives can play a role. There are efforts under way from different parts of society toward using rewards to increase donations. The U.S. 9th Circuit Court of Appeals' 2012 ruling legalizing compensation for bone marrow donations through apheresis was initiated by private individuals (32). A company prompted a 2010 European Court of Justice ruling that allowed importation of blood products obtained from compensated donors (33). Researchers and clinicians have noted that some WHO guidelines (e.g., emphasis on exclusive use of nonremunerated donors and centralizing blood collection organizations) are unintentionally adversely affecting blood collection in sub-Saharan Africa (34).

In addition to economic incentives, policy-makers should consider nonpecuniary rewards (e.g., symbolic and with social recognition) and various appeals. Debates on ethical issues around giving rewards for donations (35) should be encouraged. But there should be little debate that the most relevant empirical evidence shows positive effects of offering economic rewards on donations.

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Supplementary Materials

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EVOLUTION

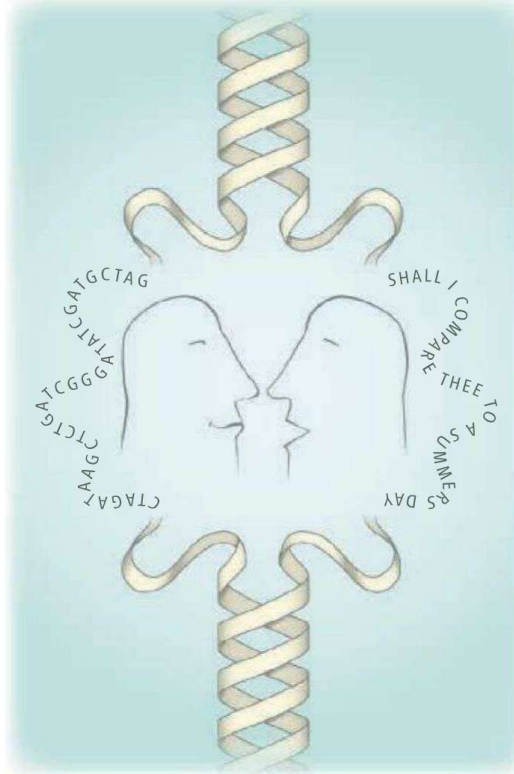
Culture, Genes, and the Human Revolution

Simon E. Fisher^{1,2} and Matt Ridley³

State-of-the-art DNA sequencing is providing ever more detailed insights into the genomes of humans, extant apes, and even extinct hominins (1–3), offering unprecedented opportunities to uncover the molecular variants that make us human. A common assumption is that the emergence of behaviorally modern humans after 200,000 years ago required—and followed—a specific biological change triggered by one or more genetic mutations. For example, Klein has argued that the dawn of human culture stemmed from a single genetic change that “fostered the uniquely modern ability to adapt to a remarkable range of natural and social circumstance” (4). But are evolutionary changes in our genome a cause or a consequence of cultural innovation (see the figure)?

Many nuanced accounts of human evolution implicitly assume that biological changes must precede cultural changes. Valender *et al.* described how alterations in size, wiring, and physiology of the human brain yielded advanced cognition, and hence a transformation of behavioral repertoires that encompassed everything from language and tool use to science and art. They posited that it is because of complex cognition that human beings are uniquely capable of cultural evolution, rather than vice versa (5). In a recent paper that elegantly emphasizes the importance of gene expression and metabolic changes in human evolution, Somel *et al.* adopt a similar view. They argue that a small number of mutations, altering the structure or expression of developmental regulators, drove the emergence of human cognitive traits to trigger a cultural explosion around 200,000 years ago (3).

This prevailing logic in the field may put the cart before the horse. The discovery of any genetic mutation that coincided with the “human revolution” (6) must take care to distinguish cause from effect. Supposedly momentous changes in our genome may sometimes be a consequence of cultural



Culture and genetics intertwined.

innovation. They may be products of culture-driven gene evolution (7).

In certain cases this is obvious. Lactase-persistence mutations did not trigger dairy farming; they spread as an evolutionary response to dairy consumption (8). The higher alcohol tolerance of Europeans relative to Asians did not prompt, but followed, greater alcohol consumption in Europe (9).

Such examples are mostly drawn from after the Neolithic revolution and the invention of agriculture. But culture-driven gene evolution may have also operated earlier in human history and could be key to understanding our origins. Wrangham's argument (10) that the invention of fire and cooking altered human gut size 2 million years ago is a case in point, positing that genetic change was contingent on prior cultural innovation.

Under the culture-driven view, many critical genomic alterations that facilitated spoken language, for example, might have spread through our ancestors after this trait emerged.

Genetic evolution may have been driven by cultural innovations during the emergence of modern humans.

That is, prior behavioral changes of the species provide a permissive environment in which the functionally relevant genomic changes accumulate. The selective advantage of a genetic change that increased language proficiency would likely be greatest in a population that was already using language.

Take the *FOXP2* gene. More than a decade ago, rare *FOXP2* mutations were implicated in an unusual inherited disorder: Affected people have problems coordinating sequences of mouth movements that underlie fluent speech, accompanied by difficulties in expressing and understanding language (11). Sequencing of versions of *FOXP2* in other primates revealed that two evolutionary changes in the protein-coding part of the gene occurred in the human lineage after it split from that of the chimpanzees (12). There was also evidence of recent Darwinian selection acting at the locus (12). The findings prompted speculation that alteration of *FOXP2* triggered the explosion of creativity marking the emergence of behaviorally modern humans (4). Further studies have suggested that the changes to the human *FOXP2* protein predated the splitting of Neandertals and modern humans several hundred thousand years ago (13). Most recently, researchers have pinpointed intronic noncoding changes at the *FOXP2* locus that arose after the split from Neandertals and that might have affected how the gene is regulated (2).

In considering the roles of *FOXP2* in human evolution, it is important to recognize that it has a deep evolutionary history. Animal studies indicate ancient conserved roles of this gene in patterning and plasticity of neural circuits, including those involved in integrating incoming sensory information with outgoing motor behaviors (14). The gene has been linked to acquisition of motor skills in mice and to auditory-guided learning of vocal repertoires in songbirds (14, 15). Contributions of *FOXP2* to human spoken language must have built on such ancestral functions.

¹Department of Language and Genetics, Max Planck Institute for Psycholinguistics, Nijmegen 6525 XD, Netherlands.

²Donders Institute for Brain, Cognition and Behaviour, Radboud University, Nijmegen 6525 EN, Netherlands. ³House of Lords, London SW1A 0PW, UK. E-mail: simon.fisher@mpi.nl

Indeed, further data from mouse models suggest that humanization of the *FOXP2* protein may have altered the properties of some of the circuits in which it is expressed, perhaps those closely tied to movement sequencing and/or vocal learning (13).

Given these findings, it seems unlikely that *FOXP2* triggered the appearance of spoken language in a nonspeaking ancestor. It is more plausible that altered versions of this gene were able to spread through the populations in which they arose because the species was already using a communication system requiring high fidelity and high variety. If, for instance, humanized *FOXP2* confers more sophisticated control of vocal sequences, this would most benefit an animal already capable of speech. Alternatively, the spread of the relevant changes may have had nothing to do with emergence of spoken language, but may have conferred selective advantages in another domain.

FOXP2 is not the only gene associated with the human revolution (3). However, it illustrates that when an evolutionary mutation is identified as crucial to the human capacity for cumulative culture, this might be a consequence rather than a cause of cultural change (8). The smallest, most trivial new habit adopted by a hominid species could—if advantageous—have led to selection of genomic variations that sharpened that habit, be it cultural exchange, creativity, technological virtuosity, or heightened empathy.

This viewpoint is in line with recent understanding of the human revolution as a gradual but accelerating process, in which features of behaviorally modern human beings came together piecemeal in Africa over many tens of thousands of years (6). Recognizing the role of culture-driven gene evolution in the origins of modern humans provides a powerful reminder of how easy it is to confuse cause and effect in science.

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CELL BIOLOGY

A Sweet Send-Off

Bertrand Kleizen and Ineke Braakman

One-third of all proteins encoded by the human genome enter the cellular secretory pathway. The first compartment, the endoplasmic reticulum (ER), is specialized for protein folding, where newly synthesized polypeptides are guided by chaperones and folding enzymes to assume a final native state. Quality control is imposed when this process fails—misfolded proteins are retained in the ER and eventually degraded, thereby keeping the cell healthy and free of protein “traffic jams.” For proteins that are glycosylated, triage decisions (and their timing) involve mannosidases and mannose-specific lectins that recognize an N-linked glycan (N-linked glycosylation site in which a nitrogen atom has been attached to an amino acid) on the polypeptide chain (1). On page 978 of this issue, Xu *et al.* (2) find that the folding of nonglycosylated proteins is terminated by a similar triage mechanism that surprisingly involves a mannose residue. This “O-mannosylation” (a sugar molecule is added to an oxygen atom in serine or threonine) may act as a cell’s timer to stop the lingering of nonglycosylated proteins that sim-

ply take too long to fold and remove them from the secretory pathway.

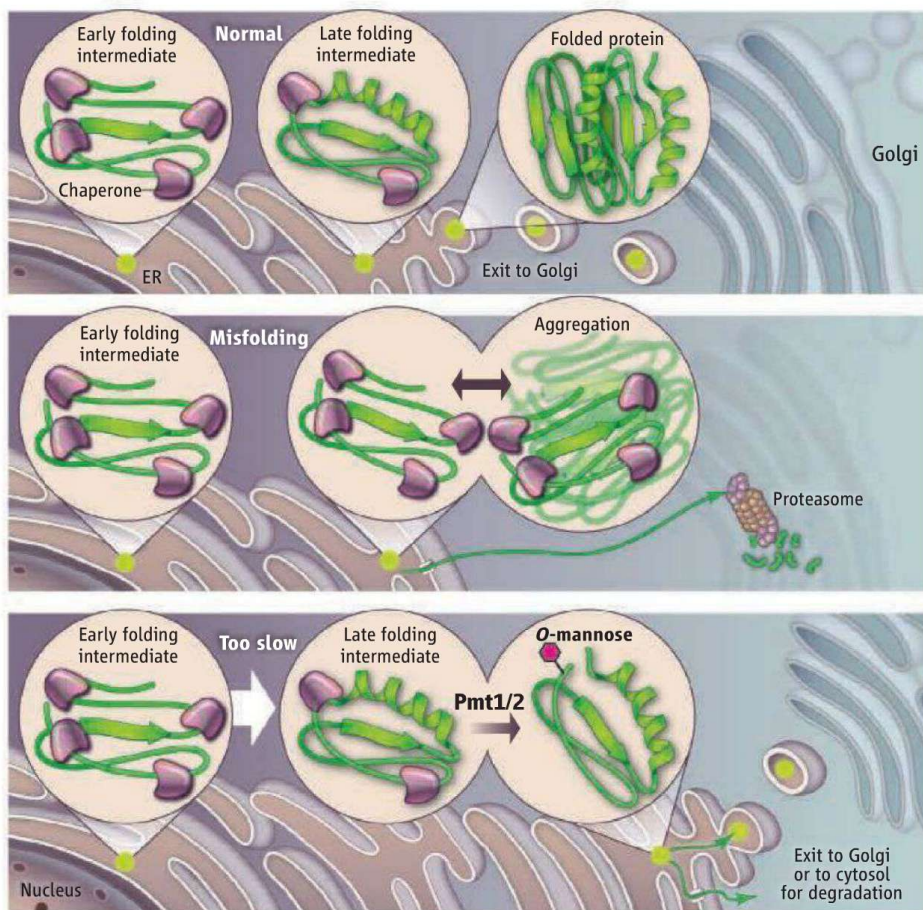
Using variants of green fluorescent protein (GFP) that are not degraded in yeast, Xu *et al.* demonstrate that only slowly or improperly folding GFPs are O-mannosylated by protein O-mannosyltransferases (Pmt1 and Pmt2) in the ER, whereas “superfolder” GFP is not. This modification leads to loss of GFP fluorescence and increased protease sensitivity, but also increased solubility and decreased aggregation, all indicative of a less folded state. Hence, O-mannosylation not only keeps the protein soluble (required for ER exit), but prevents further folding and perhaps stimulates unfolding, and flags the protein for elimination.

In the mechanism proposed by Xu *et al.*, O-mannosylation acts as a timer and stops prolonged folding of proteins, or folding that takes too many, eventually unproductive cycles. Alternatively, the Pmt1/2 O-mannosyltransferases may take on a more passive role and modify every consensus sequence that is exposed; these sequons may be shielded in early folding intermediates by extensive chaperone binding and by the compact structure in native, folded proteins. This leaves only late folding intermediates that are partially released from chaperones as sub-

strates for mannosylation. Rather than folding rate, intrinsic properties of the folding protein would determine its status as a target for Pmt1/2. The rapid O-mannosylation of peptides by mammalian orthologs of Pmt1/2 (POMT1/2) is consistent with this, although the consensus mannosylation motif reported for these enzymes (3, 4) is barely found in the GFP variants used by Xu *et al.* (either proline residues or structural determinants near threonines or serines). Whether active or passive, the timing mechanism may be stochastic. How many O-mannose residues are required for removal from the folding process is not known, nor is it clear whether O-mannosylation continues until all affinity for chaperones is lost and the fate of the protein becomes inevitable.

What makes a folding protein a substrate for O-mannosylation? The presence of serine or threonine is required (the amino acids that get modified with the sugar), but are exposed proline residues important? Proteins that sense “foldedness” in quality control usually recognize hydrophobic residues that are normally buried in the native structure, unpaired thiols, or specific N-glycans. However, even when such N-glycans are not present (5), or when a selection has been removed (6), O-mannosylation occurs. This may implicate

Cellular Protein Chemistry, Bijvoet Center for Biomolecular Research, Faculty of Sciences, Utrecht University, Netherlands. E-mail: i.braakman@uu.nl



No lingering. The model shows that chaperones assist proteins during their folding in the ER. Proteins with low folding efficiency are partially released from chaperones and become O-mannosylated by Pmt1/2. This modification increases solubility, inhibits aggregation, and prevents chaperone association and further folding. Subsequent triage steps establish whether the protein will exit to the Golgi, or to the cytosol for degradation.

O-mannosylation as a triage system for glycoproteins as well.

Can Pmt1/2 (and/or its associated proteins) sense misfolding as does the mammalian ER enzyme UDP-glucose glycoprotein transferase (UGT)? UGT sends nonnative glycoproteins back to chaperones by adding a glucose molecule to their N-linked mannose chain (7). The Pmt1/2 proteins reside in complex with a large number of chaperones (8), which would allow efficient transfer of substrates that are triaged by the chaperones. This would also accommodate the passive scenario of Pmt1/2 acting on any available consensus site that presents itself in this complex.

The role of mammalian POMTs in the ER is unclear (9). A strong disease link exists with the Walker-Warburg syndrome, which manifests as muscular dystrophy and defects in neuronal migration. Mutations in POMT1 cause abnormal glycosylation of α -dystroglycan. In skeletal muscle, α -dystroglycan is part of a complex that links the extracellular matrix to the cytoskeleton. The POMT1 mutation disrupts this interaction (10); mice lacking

POMT1 die before birth (11). The POMTs are predicted to be in the ER in mammalian cells, yet there is no evidence that they O-mannosylate proteins in that compartment. The disease symptoms and mouse phenotype suggest tissue specificity and a restricted substrate pool for the POMTs. Large-scale proteome analysis in yeast or mouse cells lacking these O-mannosyltransferases should demonstrate which protein substrates are affected most.

A fascinating aspect of the Walker-Warburg syndrome is that it places O-mannosylated wild-type α -dystroglycan at the cell surface, indicating that it gets through the secretory pathway and is released into the extracellular space. Also, in yeast, Pmt1/2 activity is crucial not only for degradation of O-mannosylated proteins but for exit of proteins to the Golgi complex (8). This is not completely surprising given that in yeast, these O-mannosyltransferases reside with folding factors and the ER's degradation machinery, as well as with proteins that transport secretory cargo to the Golgi, the next compartment in the secretory pathway.

These observations place O-mannosylation further upstream in the triage process, and it may constitute the first step in decision-making. In this model, O-mannosylation would stop protein folding and increase solubility (see the figure). The next step would determine either exit to the Golgi for productivity and function, or exit to the cytosol for degradation. Thus, those O-mannosylated proteins that exit to the Golgi have acquired a near-native conformation but are not very stable. As all proteins reside at the edge of their solubility and stability (12), O-mannosylation may help some of them escape ER quality control and move on to their functional location in or outside the cell, where partner proteins or other stabilizing conditions await them. Lectins that recognize O-mannosylated proteins would mediate either their exit from the ER or their degradation. The ER has several well-characterized mannose-recognizing proteins that are involved in the N-linked glycoprotein triage and timing process that leads to degradation. These include Htm1p and Htm2p, which are ER degradation-enhancing α -mannosidase-like proteins, and the protein Yos9p, which functions in the ER-associated protein degradation pathway. Both have been implicated in the degradation of nonglycosylated proteins as well (1). Perhaps this is where both degradation pathways merge—Pmt1/2 may feed the relatively low number of nonglycosylated proteins into the ER-associated protein degradation pathway used by the much more extensive clientele of N-linked glycoproteins.

The findings of Xu *et al.* may change our concept of quality control in the ER, as O-mannosylation may be the upstream triage event for all proteins and may be widespread. If so, this would have multiple consequences for human health and disease.

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ASTRONOMY

One Good Measure

M. R. Schreiber

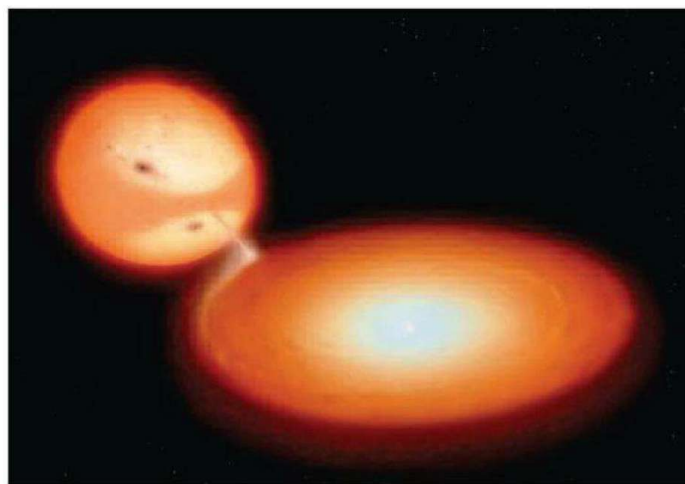
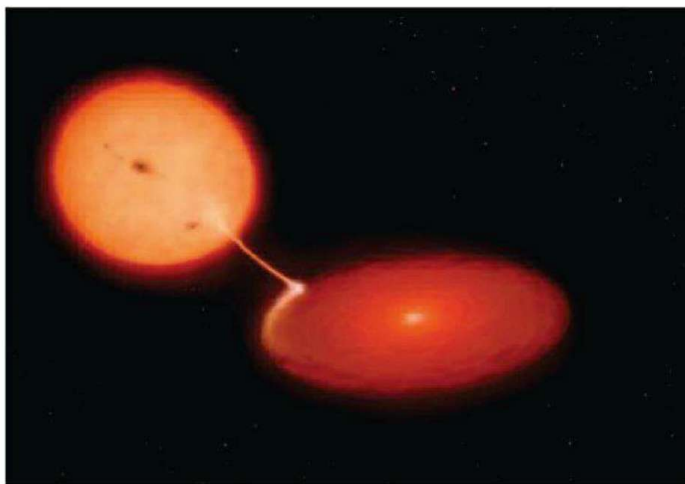
Nature shows a strong affinity for cosmic disk structures. Disks of dust and gas are the cradles of planets, the Milky Way basically is a disk of stars, and accretion disks power the engines of active galactic nuclei. About a decade ago, our understanding of accretion disks was challenged by another fundamental ingredient of astronomy—distance measurements. The

accretion. In accretion disks, viscosity does the job. Viscosity has been the great mystery of accretion disk physics because turbulence is required to explain the accretion rates derived from observations. Since the groundbreaking work of Shakura and Sunyaev (2), viscosity has been described using the viscosity parameter α , which accounts for our ignorance of the detailed nature of

Accurate distances to compact binary stars help establish a clear understanding of accretion disk evolution.

bringing us closer to revealing the mystery of viscosity (4).

The success story of the disk instability model, however, threatened to end when HST measured the distance to the best-studied accretion disk system, the dwarf nova SS Cygni, to be 159 parsecs (5), much larger than previously thought. Reproducing the observed brightness during outbursts sud-



distance to one of our main accretion disk laboratories, the binary star SS Cygni, was measured with the Hubble Space Telescope (HST) and turned out to be far too large to be in agreement with a crucial prediction of accretion disk theory. Discussions arose raising the issue that the standard accretion disk theories were perhaps wrong. On page 950 of this issue, Miller-Jones *et al.* (1) present the good news that they are not. Using radio observations, they show that the HST distance measure is wrong and that instead SS Cygni is exactly as far away as it should be according to our understanding of accretion disks. What a relief!

Accretion disks are so frequent simply because matter forced by gravitation to fall onto a compact object has difficulties getting rid of its angular momentum, and therefore cannot fall directly onto the central object but instead accumulates in a disk. Once a disk has formed, angular momentum needs to be transported outward to allow for

viscosity but facilitates simulation of accretion disk evolution and comparison of the result with observations.

Among the best test beds for accretion disk theories are compact binary stars. In these systems, a white dwarf accretes matter from a normal companion star via an accretion disk. The mass overflowing from the companion is transferred to the disk, which passes the material to the central white dwarf. The more mass is transported through the disk, the more gravitational energy is released. Some of these close binary stars, called dwarf novae, show quasi-periodic outbursts; others don't. This difference and the outburst light curves can be explained with the disk instability model (3). If the mass transfer rate is above a critical value, the disk manages to transport the same amount of material it gets from the companion. If, instead, the mass transfer rate is below this critical value, the disk becomes unstable and is forced to switch between a bright high-accretion state and a faint low-accretion state (see the figure). Comparing simulations with observed light curves allows α to be constrained,

Disk instability. Artist's impression of the two accretion stages of dwarf novae. If the rate at which mass overflows is below a critical value, the disk is unstable and switches between bright (right) and faint (left) states; otherwise, it remains in the bright state and does not produce outbursts.

denly required such a high accretion rate that the mean mass transfer rate exceeded the critical value. At 159 parsecs, models suggested that SS Cygni should not produce outbursts, but it obviously does (6). While several other difficulties of the disk instability model might be related to our ignorance of viscosity and (if the modeler is desperate enough) can be fixed by playing with the viscosity parameter α , the critical mass transfer rate is independent of α . Thus, the whole idea of accretion disks producing the outbursts was questioned by a simple distance measurement. Theoreticians seemed to be fighting a losing battle when hinting at the possibility that the distance measurement, not the model, was wrong (7, 8). The field became rather quiet and the disk instability model was used relatively little in the following decade.

Departamento de Física y Astronomía, Universidad de Valparaíso, Av. Gran Bretaña 1111, 2360102 Valparaíso, Chile. E-mail: matthias@dfa.uv.cl

It is still unclear what went wrong during the parallax measurement in 1999, but that something went wrong is now beyond doubt. The new distance measurement by Miller-Jones *et al.* is far more robust and brings SS Cygni back to where it should be—114 parsecs. With this new value, the required mass transfer rate decreases well below the critical value, and thus our concepts of accretion disk physics do not need to be revised. Moreover, it is amazing how well the disk instability model can predict the distance to SS Cygni, and it is fair to announce its comeback as one of the models most successfully linking accretion physics and observations.

These are exciting times for accretion disk research. The Atacama Large Millime-

ter Array (ALMA) is discovering the secrets of planet formation in protoplanetary disks. It seems that at least one of the mechanisms responsible for viscosity in accretion disks has been established (9). Observational efforts will improve our accretion disk laboratories: The planned Gaia mission will measure accurate distances to dozens of dwarf novae, and large HST programs on compact binaries will deliver accurate stellar parameters. Thus, we will perhaps soon be able to provide the tighter observational constraints recently requested by some of the pioneers of accretion disk research (10) and reach an adequate understanding of viscosity. Thanks to the “one good measure,” the disk instability model will play an important role in this endeavor.

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GEOPHYSICS

More Power from Below

Joseph N. Moore and Stuart F. Simmons

Geothermal heat provides sustainable energy for electricity generation and heating applications. Worldwide use of geothermal energy has increased steadily over the past few decades (1, 2), and exploration and development are ongoing at unprecedented levels in Iceland, New Zealand, East Africa, Germany, Chile, and Australia. Today, 24 countries generate electricity from geothermal energy and 78 countries use geothermal energy for direct uses. Yet, geothermal sources still represent less than one percent of global energy production. The accessibility of geothermal resources depends on temperature and depth (see the figure). What are the limitations of geothermal energy extraction, and can the use of this resource be increased?

The first commercial geothermal power plant was built in Larderello, Italy, in 1913. In this volcanically active area, hot granite lies close to the surface. Water, boiled by heat from the granite, produces the hot steam that is used to power turbines. Larderello is one of the largest geothermal electricity generators in the world, producing 594 megawatts of electrical output (MWe), sufficient to supply about 594,000 households. Even more electricity (850 MWe) is produced at The Geysers geothermal field in California, which has operated since 1960 (1). Larderello and The Geysers are vapor-

dominated, with temperatures of 240° to 300°C. A few other vapor-dominated reservoirs occur in west Java. Wells in these systems produce dry superheated steam, allowing all produced fluid to be piped directly into a turbine.

Liquid-dominated geothermal reservoirs are much more common and supply many more power stations and much more geothermal electricity than vapor-dominated reservoirs. Liquid-dominated reservoirs with temperatures >200°C are found near young volcanoes surrounding the Pacific Ocean; they also occur in rift zones and at mantle hot spots.

Flash plants are commonly used to generate electricity from these hot liquid-dominated reservoirs. The fluid can be discharged without pumping, because powerful flows develop in the well when the liquid partially flashes to steam. Most wells yield 2 to 10 MWe, but some produce >25 MWe. The steam is separated at the surface using cyclone separators; the wastewater is injected back into the reservoir, where it is reheated. Flash plants make it possible to produce steam and generate electricity from liquid-dominated reservoirs (3).

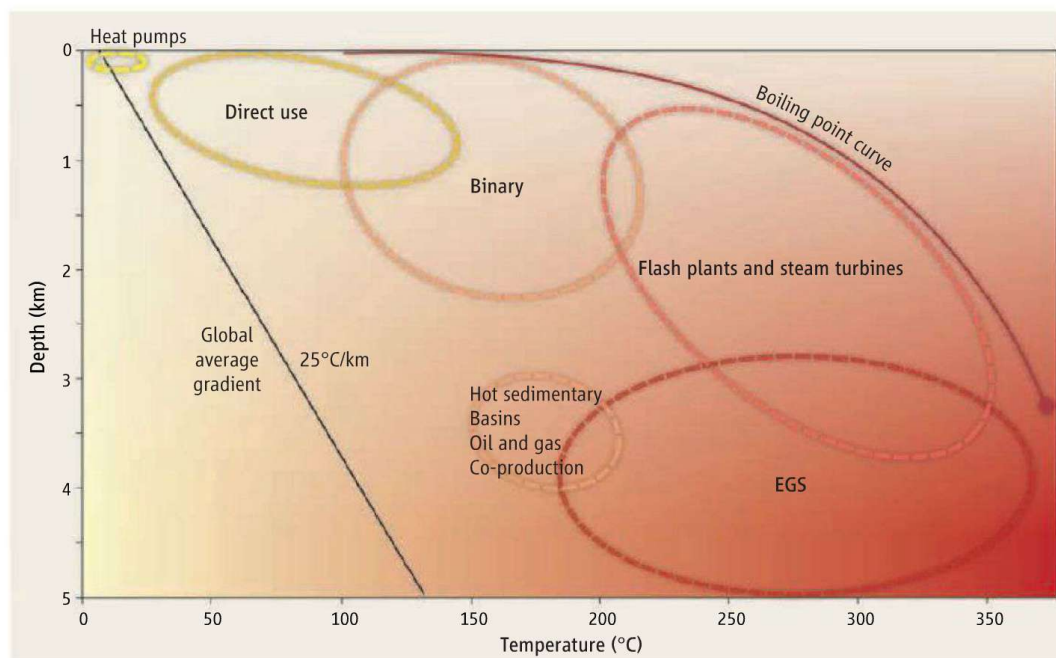
The Cerro Prieto geothermal field in Mexico (commissioned in 1973) is one of several geothermal systems in the Salton Trough, the landward extension of the Gulf of California. Here, temperatures up to 350°C have been measured. Cerro Prieto is the largest developed liquid-dominated sys-

Geothermal energy production is increasing across the world, but challenges remain.

tem at 720 MWe (2), but may be dwarfed by future development at the Salton Sea geothermal field in southern California, where resource estimates exceed 2000 MWe (4).

Liquid-dominated reservoirs with temperatures of 120° to 200°C must be pumped to produce hot water at the surface. These systems are common in extensional terrains, where the water is heated through deep circulation along faults (e.g., western United States and Turkey). The geothermal water is passed through a heat exchanger in an organic Rankine cycle binary plant containing an organic working fluid that is vaporized, passed through a turbine, and recondensed in a closed loop. These binary plants were first used to generate electricity in the Soviet Union in the late 1960s and became widely available in the 1980s (5) for small-scale electric production (up to ~50 MWe). Most new geothermal power plants brought on line in recent years in the United States are binary plants. Because the liquid is never flashed to the atmosphere, as it is in a flash plant, binary plants release no CO₂ or other gases.

Using the thermal energy from hot water directly is much more energy-efficient than generating electricity, and resources suitable for such direct use are much more widely distributed than those suitable for electric generation. Geothermal water at temperatures of 30° to 150°C is used for bathing, heating, and greenhouses in 75 countries (2). Large-scale district heating projects



Temperatures and depths for different geothermal applications. The region to the left of the ellipses is not currently economic. The boiling point curve is an upper temperature limit for geothermal systems.

have operated in Boise, Idaho, since 1892 and in Reykjavik, Iceland, since the 1930s. Today, 90% of the homes in Reykjavik are heated with geothermal water.

Ground source heat pumps exploit the lowest grade of geothermal energy at 10° to 20°C at shallow depth in regions with moderate climates. Forty-three countries have heat pump installations. The relatively constant temperature of the ground allows both space heating and cooling of homes and buildings (2). Heat pumps currently account for nearly half of all direct-use applications, and their use is increasing by ~20% per year. Direct use and heat pumps together represent a substantial energy saving equivalent to 100 million barrels of oil per year.

Few geothermal wells are drilled to depths below ~3 km because of the high cost of drilling and because permeabilities usually decrease with depth. The energy at shallower reservoir depths (1 to 3 km) is only a small fraction of the total recoverable heat at deeper depths that can be reached using standard oil and gas drilling technology (down to 10 km). Capturing even 2% of the thermal energy at depths of ~3.5 to 10 km could provide 2000 times the annual energy use in the United States (6, 7). To access this heat, reservoirs capable of sustaining commercial fluid flow rates for 20 to 30 years must be created in relatively impermeable hot rock. The cooled, re-injected water must be reheated in the reservoir before it is produced again.

Permeability creation in these Enhanced Geothermal Systems (EGS) relies on opening existing fractures through water injection. The technologies used in EGS development have been adapted from oil and gas extraction techniques, but the target geologic formations are deeper, no toxic chemicals are used, and the risk of adverse environmental impacts is much lower. Once a well is drilled and stimulated, additional wells can be directionally drilled to target the fractured reservoir.

Large-scale EGS development will require adequate fracture development to afford reservoir-scale permeability enhancement. Small electricity-generating plants in EGS reservoirs have been built in the Rhine Graben at Soultz-sous-Forêts in France (1.5 MWe) and at Landau (3 MWe) and Insheim (4.5 MWe) in Germany (8). One of the most exciting EGS projects lies in a remote part of South Australia, where radiogenic granites with temperatures up to 250°C at ~4 km depth underlie an area covering ~2000 km² (9). EGS resources are also being investigated at several sites in the United States.

Hot sedimentary basins are a promising target for large-scale, geothermal development within the next decade. Although few geothermal wells have been drilled into these basins, many oil and gas wells have. Measurements from these wells indicate that temperatures above 150°C and high permeabilities exist at depths of 3 to 4 km. Because of their lateral extent (>100 km²), individ-

ual basins have the potential to generate 100 to 1000 MWe (10). Worldwide, deep sedimentary basins and EGS reservoirs hold the greatest potential for new development; hot water produced with oil and gas is another substantial resource (7).

The geothermal plants at Larderello, Wairakei, Cerro Prieto, and The Geysers prove that hot geothermal reservoirs can produce sustainably for many decades, and in many parts of the world, this type of conventional resource (<3 km depth) attracts considerable ongoing investment. However, the awareness and popularity of geothermal heat pumps makes this the fastest growing sector of geothermal utilization. Future development will take advantage of the vast

untapped thermal resource underlying most continental regions at greater depths of 3 to 10 km. Understanding how to develop and manage heat transfer and fluid flow in these deep geologic environments over commercially viable periods (>25 years) remain top priorities for research and development.

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CHEMISTRY

All Change for Nanocrystals

Maria Ibáñez and Andreu Cabot

Until recently, to prepare nanocrystals of a new material, scientists searched their shelves for the appropriate molecular precursors, surfactants, and solvents. They then optimized the reaction conditions for the atoms to self-assemble into monodisperse nanocrystals (1). This approach is being replaced by a simpler strategy, in which preformed nanocrystals serve as templates to produce nanoparticles with a different composition through chemical transformation. On page 964 of this issue, Oh *et al.* (2) report a powerful mechanism that allows the composition of oxide nanoparticles to be transformed in solution and at low temperatures.

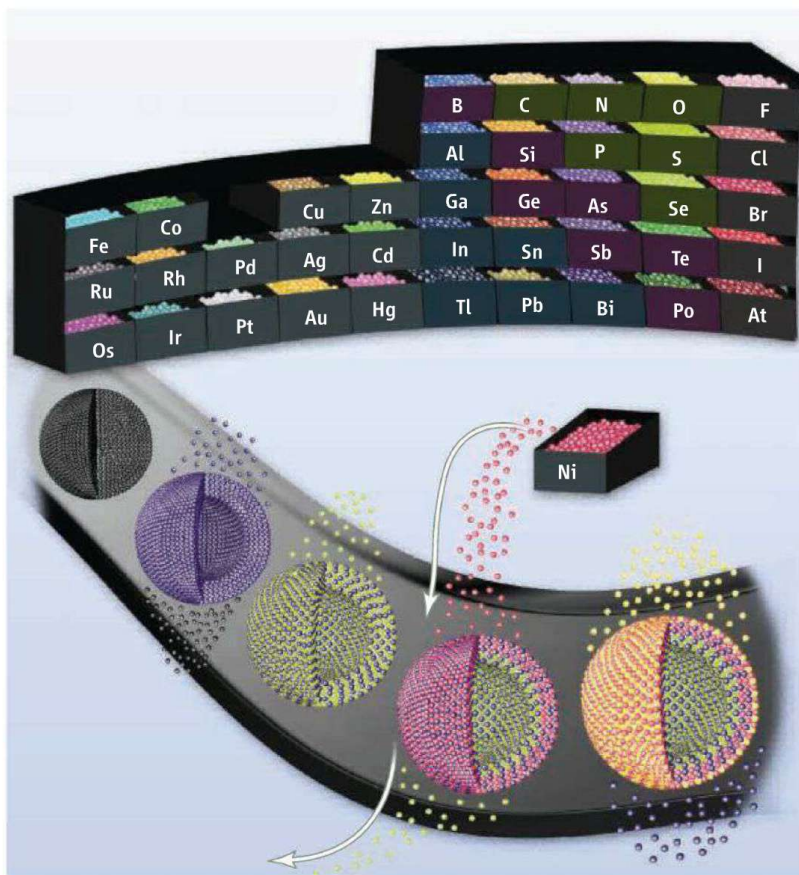
The report adds to a handful of solution-based mechanisms to chemically transform nanomaterials. These mechanisms allow atoms to be easily yet precisely incorporated, removed, or replaced from preformed template nanoparticles by means of oxidation, reduction, alloying, or atomic exchange reactions (3–8). In ionic nanocrystals, cation exchange can be driven by differences in solvation energies between the ions in the nanocrystal template and the ions in solution. The different ion solubilities can be controlled by adding selective coordinating species to the solution (5, 9, 10). In metal nanocrystals, atomic exchange reactions can be driven by reduction potential differences between the metal in the template and the metal ions in solution. This mechanism is known as galvanic replacement and involves a redox reaction: When placing a nanocrystal in a solution containing ions of a metal with a

higher reduction potential, the surface of the nanoparticle template oxidizes, and its metal ions dissolve. The released electrons reduce the ions from the new element in solution, which deposit at the template nanoparticle surface.

Oh *et al.* now extend the galvanic replacement mechanism to ionic compounds. In oxide nanocrystals, galvanic replacement takes place through a redox-couple reaction between multivalent metallic ions. As an example, Oh *et al.* replace the higher-oxidation state ions in manganese oxide nanocrystals with lower-oxidation state iron ions from solution.

Although the driving forces are different, atomic diffusion is a key parameter in all these chemical transformation reactions. Chemical transformation tools provide com-

A chemical method allows the composition of oxide nanocrystals to be changed while maintaining their size and shape uniformity.



Multicomposition nanostructures. Oh *et al.* report a mechanism that can be used to change the composition of oxide nanocrystals. This and related methods are expanding the range of nanocrystal compositions that can be created, with potential applications in fields ranging from electronics and catalysis to biotechnology.

plete control over composition only within the atomic diffusion length. In nanocrystals, high surface-to-volume ratios make the entire lattice accessible to diffusion. The particle size range in which these tools can be used effectively strongly depends on the material, but it can reach up to hundreds of nanometers in particular systems (11).

This set of tools enables the production of porous and hollow nanostructures that cannot be made with other existing approaches. By means of the Kirkendall effect, hollow nanoparticles can be created by vacancy accumulation during the diffusion of template atoms through the shell to react with a new element in solution (3, 11). Hollow nanostructures can also be produced by the galvanic replacement reaction (7). Unlike with the Kirkendall effect, galvanic replacement does not require differential atomic diffusion

between the template and the new element, because only charges, not ions, need to move through the shell. In this case, dissolution of the inner template is assisted by shell pinholes, which provide avenues to transport the dissolved ions.

Multicomponent nanomaterials with compositional and morphological complexity are also within reach of these synthetic tools. Nanostructures with a potentially unlimited variety of compositions may be produced through successive use of different partial chemical conversion reactions (10, 12, 13). Such precise control of nanomaterial composition opens the door to fundamental studies with accurately controlled systems and to the production of nanomaterials with carefully engineered functional properties. These nanomaterials are of interest for appli-

cations in fields ranging from biotechnology to energy and electronics. As an example, Oh *et al.* show how porous multimetallic oxide nanostructures can be produced in a very simple way with a high level of control over composition. They further demonstrate that such porous multimetallic oxides are excellent anode materials for lithium ion batteries. This precise control over material properties is accompanied by the relatively straightforward scalability, low cost, and ease of technological application inherent to solution synthesis.

Oh *et al.*'s study provides one more tool to control material composition at the

nanoscale. Researchers must now learn to master its use. The reliable and large-scale production of complex multicomponent nanostructures requires compositional or crystallographic selectivity of the chemical transformation and that partial reactions be self-limited to overcome potential reactant gradients. For industrial applications, scientists must also deal with the recycling or reuse of the sacrificed ions. Most important, we will need to sharpen our imagination. The limitation will soon no longer lie in producing the target nanomaterial but in designing them to ever more challenging performance specifications.

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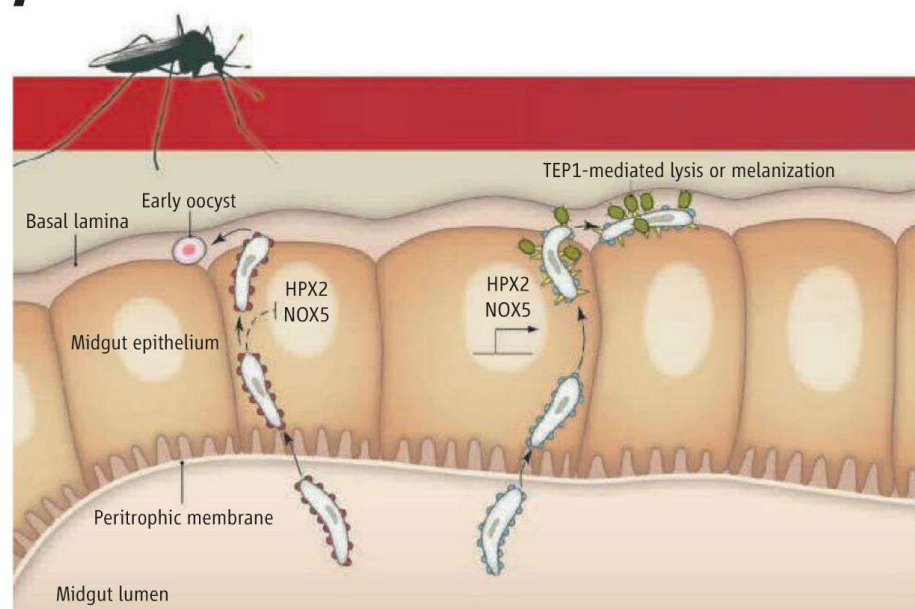
MICROBIOLOGY

Unveiling the Malaria Parasite's Cloak of Invisibility?

Nisha Philip and Andrew P. Waters

Several insects, particularly mosquitoes, are responsible for the transmission of many viral, protozoan, and even worm infections. Previously, insects were considered passive pathogen carriers. But sophisticated cross-talk exists between the pathogen effectors and the immune system of the insect vector. As in vertebrates, the insect's first line of defense is a sophisticated innate immune response (IIR), employing pattern recognition molecules to detect pathogens and shape the anti-pathogen response. IIR involves humoral and cellular responses (1), including phagocytic cells and complement-like systems, which result in the activation of effector molecules—for example, thioester-containing protein 1 (TEP1) (2)—that cause lysis or encapsulation of the parasite through melanization. IIR may also trigger free-radical release, causing lethal damage to the pathogen. Despite a fairly detailed picture of how a mosquito responds to pathogen infection (3, 4), parasite targets and mechanisms of mosquito immune evasion by parasites are largely unknown. On page 984 of this issue, Molina-Cruz *et al.* (5) identify a gene in the human malarial parasite, *Plasmodium falciparum*, that mediates evasion of the mosquito IIR.

Wellcome Trust Centre for Molecular Parasitology, Institute of Infection, Immunity and Inflammation, University of Glasgow, College of Medical, Veterinary, and Life Sciences, 120 University Place, Glasgow G12 8TA, UK. E-mail: andy.waters@glasgow.ac.uk



Evasion mosquito immunity. Fertilized parasite gametes develop into a motile ookinetes, which penetrate the insect gut epithelium. Parasite exposure results in activation of epithelial heme peroxidase (HPX2) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 5 (NOX5), which are enhancers of midgut epithelial nitration and antiplasmodial activity. Epithelial nitration might lead to molecular tagging of the ookinete (yellow) and target the parasite for destruction by the TEP1-mediated complement system. Parasites expressing certain *Pfs47* alleles (red) remain undetected or suppress the mosquito nitration response, thereby escaping the complement-mediated antiplasmodial activity. Ookinete surface markers: right, blue (PFS47 recognized by the mosquito IIR); left, red (PFS47 evading IIR); top, green (parasites tagged for destruction).

Plasmodium spp. are the single-cell protozoa that cause malaria, and *P. falciparum* is the major human pathogen responsible for ~1 million deaths each year in Africa alone. *Plasmodium* is transmitted by female, generally anopheline mosquitoes that require blood meals to mature their eggs. Once they are ingested by the mosquito, the parasite under-

goes a remarkable developmental progression: Specialized sexual precursor cells (male and female gametocytes) that were circulating in the blood of an infected mammalian host are immediately activated to form gametes. The gametes then fuse into a zygote that develops within 18 to 24 hours into a sausage-shaped motile ookinete, which is the truly

infectious agent to the mosquito. The ookinete burrows through the mosquito's midgut wall, forming an oocyst on the far (basolaminar) side within which >10,000 sporozoites are formed over the following 10 days. Upon oocyst rupture, sporozoites migrate through the hemolymph to the salivary glands for delivery during the next blood feed. To eliminate the parasite early in its life cycle, the mosquito IIR targets the ookinete as it passes through the midgut wall and thereafter develops into the oocyst. Indeed, the parasite suffers disproportionate loss in absolute numbers at the ookinete-oocyst transition (6).

To understand how the parasite avoids the insect IIR, Molina-Cruz *et al.* performed a forward genetics screen that implicated regions of parasite chromosomes 4, 7, and 13. Focusing on chromosome 13, they homed in on a key factor in immune escape as a highly structured glycosylphosphatidylinositol-anchored membrane protein PFS47, a member of the 6-cysteine (6-Cys) family of surface and secreted proteins that has been best characterized in *Plasmodium*. The 10 members of the 6-Cys family are expressed during different stages of the *Plasmodium* life cycle and are thought to mediate extracellular interactions through protein-protein interactions (7). Whereas S47 in the rodent malaria parasite is centrally involved in gamete fusion (8), its role in *P. falciparum* is unclear (9). Using various approaches, Molina-Cruz *et al.* demonstrate that PFS47 is exquisitely evolved to provide protection against the complement pathway (TEP1)-mediated innate immune response in the local mosquito population. In *P. falciparum* genetically engineered to lack PFS47, the IIR recognizes and eliminates the parasites by TEP1-mediated lysis (which may also be associated with melanization) facilitated by the free-radical nitration-based response (10). In addition, there is clearly an "arms race" in progress, as the *pfs47* allele from a Brazilian strain cannot mask an African strain from the African mosquito immune response. *pfs47* alleles also exhibit a high rate of highly localized nonsynonymous polymorphism that is associated with evasion of IIR and is located in a different region of the protein than focused polymorphisms associated with fertilization (8).

Molina-Cruz *et al.* propose that PFS47 represses the nitration response, preventing recognition by the mosquito complement system. Consequently, it would be interesting to examine whether absence of PFS47 increased TEP1 binding to ookinetes, and how PFS47 aids in dodging the mosquito immune response. Polymorphisms notwithstanding, PFS47 is unlikely to be the direct

target of an immune attack. Whether the protein has a role in passive evasion by making the parasite invisible to the mosquito immune system, acts as a decoy to prevent recognition of another surface protein, or anchors the decoy protein should be investigated. It may be that PFS47 actively interferes with the mosquito immune response, in which case IIR proteins might be covarying. Although PFS47 is clearly central, the role of factors encoded by genes on the other two regions on chromosomes 4 and 7 implicated in the phenomenon should be examined.

The study by Molina-Cruz *et al.* opens a new window into vector-parasite interactions, and future studies should elucidate the molecular and biochemical mechanisms of PFS47 action on the mosquito immune system. Per-

haps with the merest tweak, the mosquito IIR can be potentiated to the point that malaria's cloak of invisibility becomes all too apparent.

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IMMUNOLOGY

Antigen Processing Takes a New Direction

Nilu Goonetilleke and Andrew J. McMichael

A vaccine that protects against SIV in monkeys elicits a new kind of T cell response.

T lymphocytes expressing $\alpha\beta$ T cell receptors can be divided into those that express either CD8 or CD4 glycoproteins on their cell surface. Major histocompatibility complex (MHC) molecules on antigen-presenting cells present small fragments of antigenic proteins (epitopes) to T cells, and here again is a distinction: MHC class I (MHC-I) present to CD8⁺ T cells, whereas MHC class II (MHC-II) present to CD4⁺ T cells. Classical CD8⁺ T cells kill target cells, whereas CD4⁺ T cells exhibit effector and regulatory functions through cytokine secretion. On page 940 of this issue, Hansen *et al.* (1) challenge these paradigms of T cell antigen recognition and function and raise the possibility of achieving distinct T cell responses through the genetic manipulation of vaccine vectors.

Hansen *et al.* characterized the T cell response in monkeys elicited by a laboratory-derived recombinant rhesus monkey cytomegalovirus (strain 68-1 RhCMV) engineered to express simian immunodeficiency virus (SIV) genes. It was previously

observed that when administered as a vaccine to monkeys, this recombinant vector generated an unprecedented level of protection to SIV challenge, with 50% of immunized animals completely clearing SIV, an effect mediated by CD8⁺ T cells (2). Given the failure to find vaccines that induce protective CD8⁺ T cell responses against HIV-1 in humans, this observation warranted close scrutiny. The key finding of Hansen *et al.* is that strain 68-1 RhCMV elicited CD8⁺ T cell responses that target SIV epitopes that were completely different from those generated by SIV infection itself, by other virus-based vectors, or by wild-type RhCMV expressing SIV genes.

CD8⁺ T cells responding to an infecting virus typically focus on a small number of epitopes. The uneven expansion of these responses in vivo results in a hierarchy of immunodominance (see the figure). This phenomenon is not fully understood but may reflect competition between T cells of different specificities responding to limiting amounts of different peptide-MHC molecules on the surface of antigen-presenting cells. Immunodominant T cell responses detect and kill the most virus-infected cells, therefore exerting greater immune pressure on the infecting virus (3). T cell immuno-

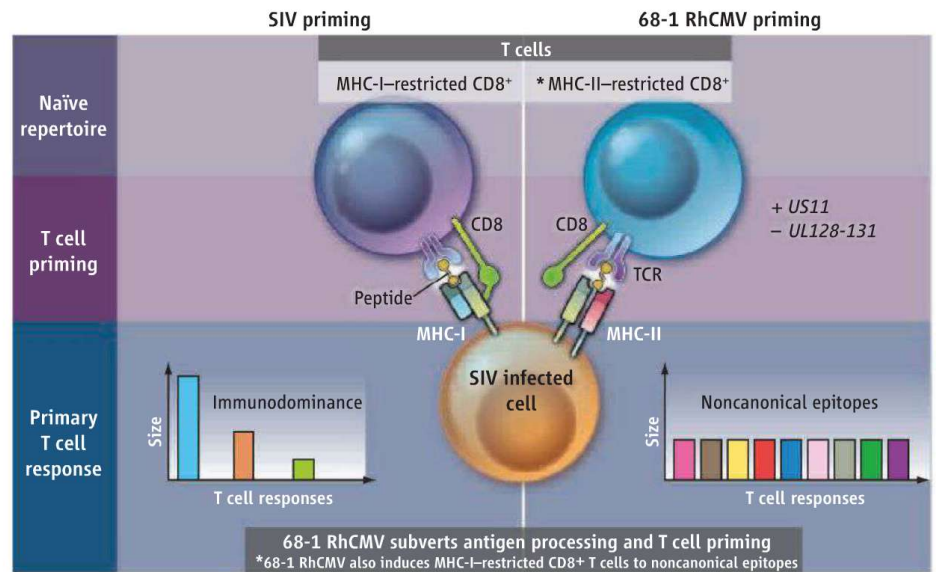
Weatherall Institute of Molecular Medicine, University of Oxford, Headington, Oxford OX3 9DS, UK. E-mail: andrew.mcmichael@ndm.ox.ac.uk

dominance may also limit cross-reactivity to self-antigens and minimize the risk of autoimmunity (4).

By contrast, strain 68-1 RhCMV elicited CD8⁺ T cell responses to a very broad range of SIV epitopes presented by both MHC-I and MHC-II. The former did not include canonical epitopes typically targeted in CD8⁺ T cell responses to SIV proteins. The latter, constituting two-thirds of the response, recognized longer peptides that were frequently presented by several different MHC-II allotypes. Both MHC-I- and MHC-II-restricted CD8⁺ T cells were of equal magnitude with no clear immunodominance hierarchy, quite unlike other virus infections, including wild-type RhCMV. Critically, the T cells elicited by strain 68-1 RhCMV recognized and lysed SIV-infected cells, indicating that SIV-infected cells can present these epitopes but normally do not prime MHC-II-restricted CD8⁺ T cells. This means that the difference between the CD8⁺ T cells induced by strain 68-1 RhCMV and the “normal” CD8⁺ T cell responses to canonical MHC-I presented epitopes is set at the stage of immunological priming of naïve T cells.

By comparing strain 68-1 RhCMV and wild-type RhCMV with a series of RhCMV vectors containing gene deletions, Hansen *et al.* show that the rhesus CMV gene *Rh189* suppressed induction of MHC-I-restricted CD8⁺ T cell responses to canonical epitopes. Human CMV *US11* is the ortholog of rhesus CMV *Rh189* and is known to target nascent MHC-I molecules for degradation (5, 6). This may influence priming of CD8⁺ T cells (7), but Hansen *et al.* argue that other unknown functions of *US11* are likely to be involved. Induction of the broad MHC-I- and MHC-II-restricted CD8⁺ T cell responses targeting the noncanonical epitopes by strain 68-1 RhCMV depended on the absence of the RhCMV orthologs of *UL128-131*. These genes broaden the tropism of rhesus and human CMV, and their loss seems to subvert T cell priming. How limiting the tropism of strain 68-1 RhCMV results in the induction of such broad and MHC-II-restricted CD8⁺ T cell responses requires further investigation.

MHC-II-restricted CD8⁺ T cells have been described previously, although mostly in special circumstances of genetically modified mice (8). The substantial and reproducible breadth of the MHC-II-restricted CD8⁺ T cell responses to SIV infection that is induced by strain 68-1 RhCMV in monkeys suggests that this type of T cell must be relatively abundant in the naïve repertoire before priming with the vaccine. Although it



Less restricted. A recombinant viral vector that expresses SIV genes (strain 68-1 RhCMV) expands the repertoire of viral epitopes that CD8⁺ T cells respond to. The presence or absence of specific genes encoded by the CMV vector itself allows the subversion of CD8⁺ T cells from a narrow response to a limited number of epitopes presented by MHC-I molecules (left) to a broader range of epitopes presented by MHC-II molecules.

is possible that such T cells may be elicited in other infections, previous fine mapping of antiviral CD8⁺ T cell responses has almost always identified short 8- to 11-amino acid epitopes that typically bind to MHC-I. Why MHC-II-restricted CD8⁺ T cell responses are not more commonly expanded after infection is unclear. It is possible that this process is highly regulated because the promiscuous binding of MHC-II-restricted CD8⁺ T cells could increase cross-reactivity and lead to autoimmunity.

Do the findings of Hansen *et al.* explain the protection offered against SIV challenge by recombinant strain 68-1 RhCMV? It was previously shown (2) that the magnitude of CD8⁺ T cell responses induced by the recombinant strain, prior to challenge by SIV infection, correlated with SIV control and eradication. No doubt the greater CD8⁺ T cell breadth induced by strain 68-1 RhCMV contributed to the high magnitudes observed. These observations contrast with vaccine studies that used viral vaccine vectors other than SIV, such as adenoviruses, which elicited classical MHC-I-restricted CD8⁺ T cell responses (9). The expanded memory CD8⁺ T cell response after challenge correlated with lower SIV loads but without complete control (9). The incomplete virus control following induction of classical CD8⁺ T cell responses, with narrow immunodominance hierarchies, could be explained in part by virus variability and escape after SIV challenge (10, 11), as happens in HIV-1 infection (12).

Whether both MHC-I- and MHC-II-restricted CD8⁺ T cells are necessary for the virus control seen with the strain 68-1 RhCMV vaccine is still to be determined. Why only 50% of challenged monkeys were protected (2) is also a crucial question. Given that the critical RhCMV genes that control CD8⁺ T cell targeting have homologs in human CMV, equivalent vaccine vectors could be engineered to induce similar broad T cell responses in humans against HIV-1 and other viruses. An alternative approach would be to unravel the mechanisms behind the immunogenicity of strain 68-1 RhCMV and to find other ways to elicit the same T cell responses; this would take longer to achieve but would avoid the possible risks of a persisting CMV-based vaccine. Given the recent failure of the HVTN505 DNA and recombinant adenovirus-5 vaccine regimen, which largely induces classical MHC-I-restricted CD8⁺ T cells, to protect against HIV-1, these findings of Hansen *et al.* could not be more timely.

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RETROSPECTIVE

François Jacob (1920–2013)

Lucy Shapiro¹ and Richard Losick²

François Jacob died on 19 April in Paris at the age of 92. We have lost one of the great architects of modern biology, whose legacy touches all aspects of gene regulation from viruses and bacteria to humans, as well as in human diseases such as cancer. To an astonishing degree, his insights and elegant experiments established the framework for understanding the mechanisms that underpin gene control in every decision a cell makes on its journey through the cell division cycle, in embryogenesis, in tissue differentiation, and in the formation of a multicellular organism. He was not only a visionary scientist but also a humanist with extraordinary perception of the workings of the scientific mind and the literary ability to convey his insights with grace and compassion.

Jacob wrote and talked about “day science” and “night science.” He described day science as how we present our discoveries in seminars and papers, as a linear progression of observations and scientific design to a “voilà” conclusion. Night science is how the discovery process really happens, in its messy, intuitive, questioning progression, where we construct and then demolish hopeful hypotheses, “fighting a lot with yourself.” Most important to this process is how one formulates questions that then drive the design of the experiment. He spoke eloquently of the tool box of life, like engineer’s parts on a shelf, which through evolution undergo arrangements and rearrangements to construct complex genetic regulatory circuits that result in the myriad of Earth’s life forms.

François Jacob was born in 1920 in Nancy, France. His journey from a bourgeois Parisian life as a medical student to his immersion in phage genetics with André Lwoff at the Institut Pasteur included a hiatus imposed by the invasion of France by Nazi Germany. Jacob managed to get out of Paris and eventually join Charles de Gaulle in England. He

fought with the Free French Army in Europe and North Africa until 1944 when he was seriously injured, spending many months recovering from wounds that would end his chosen career as a surgeon and that would plague him for the rest of his life. At loose ends, he tried journalism and acting, but completed his medical degree in 1947 while looking for other ways of constructing a new life. He eventually learned of the research going on in the “attic” at the Institut Pasteur and decided that he would join the world of phage and bacteria. Every month he would apply to work with Lwoff but was summarily rejected. Finally, Lwoff relented, telling him excitedly about his discovery of prophage induction and that he needed someone to work on it. Jacob responded, “I’m dying to work on induction,”

without a clue about what it meant. In 1950, Jacob joined the “attic,” with Lwoff at one end of a long corridor and Jacques Monod at the other. Just 15 years later, Jacob, Monod, and Lwoff would be awarded the 1965 Nobel Prize in Physiology or Medicine for “their discoveries concerning genetic control of enzyme and virus synthesis.”

Jacob earned a doctorate in science in 1954 at the Sorbonne, focusing on the concept of a provirus. He observed that bacteriophage—a virus that infects bacteria—could integrate its genome into that of a host bacterium, remain dormant as a provirus (prophage), and have its genome passed on to bacterial offspring. When the provirus becomes active again, the virus enters a lytic cycle in which its genome is expressed and new virus is produced to infect other bacteria.

Experimental results from Élie Wollman, Jacob, and Lwoff on lysogeny and immunity, and from Monod on lactose induction of β -galactosidase synthesis in bacteria were merged in the “Big Synthesis,” which postulated that the two seemingly unrelated systems were controlled by a common mechanism involving repressors that inhibit gene activity. That is, genes are controlled by dedicated macromolecules (initially thought to be RNA, but later shown to be protein, although

The French Nobel laureate ushered in the field of molecular genetics by defining the regulated cellular pathway that translates DNA into proteins.

we now know that both control genes) that regulate their activity. Monod held that all regulation must be mediated by repressors, whereas Jacob believed that activators could also be part of gene-control pathways, an idea that later proved to be correct.

The work with β -galactosidase resulted in the identification of the lactose operon (a cluster of three adjacent genes that controls the transport and metabolism of lactose in bacteria) and led the Pasteur team to postulate the existence of “factor X” as an intermediate between the gene and the ribosome to produce protein. These ideas, as well as experiments by Elliot Volkin and Lazarus Astrachan, pointed to factor X being messenger RNA, which was demonstrated by Sydney Brenner, Matthew Meselson, and Jacob during a 1-month visit to Max Delbrück at the California Institute of Technology in 1960.

Studying conjugation (the transfer of genetic material by direct cell-to-cell contact) in *Escherichia coli*, Wollman and Jacob demonstrated that the order of genes on the chromosome is linear and that this order undergoes a circular permutation during chromosome transfer between strains. This led to the surprising but correct conclusion that the *E. coli* chromosome must be circular.

Every discovery by this remarkable man has its imprint on the logic and reasoning that are used to this day to understand life. From early on in his time at the Pasteur, Jacob was fixated on understanding how a pair of cells with the same DNA could be programmed to turn into two different kinds of cells. This quest ultimately led him to switch from *E. coli* and phage lambda to an organism with “eyes, a soul and plenty of sex”—the mouse. The combination of genetics and the possibility of a cell culture system led him to propose an interdisciplinary “Institute of the Mouse” at the Pasteur to study developmental regulation of a complex being. Although the institute never materialized, Jacob went on to use the mouse as a model system to explore the relationship between embryonic development and cancer, “clearly a disease of regulation.”

Throughout his life in science, Jacob knew that he was involved in the “great adventure of the century,” and his eloquent books about this adventure are a living testimony to his legacy.



¹Developmental Biology, Stanford University, Stanford, CA 94305, USA. ²Molecular and Cellular Biology, Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA. E-mail: shapiro@stanford.edu; losick@mcb.harvard.edu

Cytomegalovirus Vectors Violate CD8⁺ T Cell Epitope Recognition Paradigms

Scott G. Hansen, Jonah B. Sacha, Colette M. Hughes, Julia C. Ford, Benjamin J. Burwitz, Isabel Scholz, Roxanne M. Gilbride, Matthew S. Lewis, Awbrey N. Gilliam, Abigail B. Ventura, Daniel Malouli, Guangwu Xu, Rebecca Richards, Nathan Whizin, Jason S. Reed, Katherine B. Hammond, Miranda Fischer, John M. Turner, Alfred W. Legasse, Michael K. Axthelm, Paul T. Edlefsen, Jay A. Nelson, Jeffrey D. Lifson, Klaus Früh, Louis J. Picker*

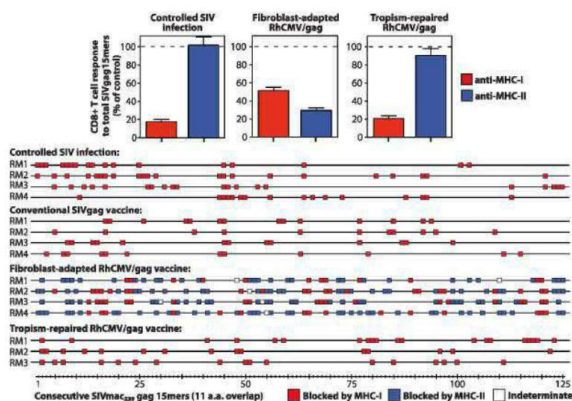
Introduction: CD8⁺ T cell responses focus on a small fraction of total pathogen-encoded peptides, which are similar among individuals with shared major histocompatibility complex (MHC) alleles. This focus can limit immune control of genetically flexible pathogens, such as HIV and SIV, because CD8⁺ T cells in most infected subjects do not target sequences required for pathogen fitness, resulting in viral escape. Although a vaccine capable of broadening or redirecting CD8⁺ T cell epitope targeting to prevent viral escape would be highly advantageous, it remains unclear whether this targeting can be diverted from its default pattern during priming.

Methods: We used intracellular cytokine analysis to compare the epitope targeting of SIV-specific CD8⁺ T cell responses in rhesus macaques with controlled SIV infection or after vaccination with either conventional SIV vaccines or rhesus cytomegalovirus (RhCMV) vectors. RhCMV vectors have been associated with stringent control of SIV challenge in the absence of protective MHC alleles.

Results: Fibroblast-adapted RhCMV/SIV vectors elicited SIV-specific CD8⁺ T cells that failed to target any canonical epitopes associated with SIV infection or conventional SIV vaccination. Instead, they recognized distinct epitopes characterized by extraordinary breadth (greater than that of conventional vaccines by a factor of >3), MHC class II (MHC-II) restriction (63% of epitopes), and high promiscuity (epitopes common to most or all responses in vaccinated macaques). These unconventionally targeted CD8⁺ T cell responses recognized autologous SIV-infected cells, indicating that processing and presentation of the unconventional epitopes

is CMV-independent. However, CMV gene expression was responsible for directing epitope specificity of CD8⁺ T cells during priming. The induction of canonical SIV epitope-specific CD8⁺ T cell responses was specifically suppressed by expression of the *Rh189/US11* gene, and the promiscuous MHC-I- and MHC-II-restricted CD8⁺ T cell responses occurred only in the absence of the *Rh157.4–.6/UL128–131* genes involved in CMV tropism for nonfibroblasts.

Discussion: These findings suggest that CD8⁺ T cell recognition is more flexible than had been thought, and that the focused epitope recognition profiles of conventional CD8⁺ T cell responses may be primarily restricted by immunoregulation during priming (which can be subverted by CMV) rather than by intrinsic



Fibroblast-adapted RhCMV/gag vectors elicit MHC class II-restricted CD8⁺ T cells, greatly expanding the breadth of the response. (Top) Differential inhibition of SIVgag-specific CD8⁺ T cells from SIV⁺, fibroblast-adapted RhCMV/gag vector-vaccinated, and tropism-repaired RhCMV/gag vector-vaccinated rhesus macaques by MHC-I versus MHC-II blockade. (Bottom) Responses to consecutive SIVgag 15mer peptides in the indicated animals, classified by sensitivity to MHC-I versus MHC-II blockade.

limitations in antigen processing/presentation or in T cell receptor repertoire. The ability of CMVs with different genetic modifications to differentially elicit CD8⁺ T cell responses with divergent patterns of epitope recognition raises the possibility of a CMV vector-based vaccine platform with programmable CD8⁺ T cell epitope targeting, including vectors that can selectively elicit CD8⁺ T cell responses targeting conventional or unconventional epitopes. Because the latter would be unaffected by escape mutations arising during natural infection, these vectors would be well suited for therapeutic vaccine applications.

READ THE FULL ARTICLE ONLINE
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FIGURES IN THE FULL ARTICLE

Fig. 1. Lack of epitope overlap between RhCMV vector-elicited and conventional SIV-specific CD8⁺ T cell responses.

Fig. 2. RhCMV vector-elicited and conventional SIVgag-specific CD8⁺ T cell responses differ in epitope breadth and promiscuity.

Fig. 3. Most RhCMV vector-elicited CD8⁺ T cell responses are inhibited by MHC-II blockade.

Fig. 4. Multiple MHC-II allomorphs can present type 2 epitopes to RhCMV/gag vector-elicited CD8⁺ T cells.

Fig. 5. RhCMV/gag vector-elicited CD8⁺ T cells show similar function regardless of MHC-I versus MHC-II restriction.

Fig. 6. RhCMV vector-elicited CD8⁺ T cells recognize SIV-infected cells via both MHC-I and MHC-II antigen presentation.

Fig. 7. *Rh189 (US11)* gene prevents canonical epitope recognition.

Fig. 8. Unconventional CD8⁺ T cell targeting is restricted to responses elicited by fibroblast-adapted RhCMV lacking UL128–131 ortholog expression.

SUPPLEMENTARY MATERIALS

Figs. S1 to S12
 Tables S1 to S3

Vaccine and Gene Therapy Institute and Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR 97006, USA.

*Corresponding author. E-mail: pickerl@ohsu.edu

Cytomegalovirus Vectors Violate CD8⁺ T Cell Epitope Recognition Paradigms

Scott G. Hansen,¹ Jonah B. Sacha,¹ Colette M. Hughes,¹ Julia C. Ford,¹ Benjamin J. Burwitz,¹ Isabel Scholz,¹ Roxanne M. Gilbride,¹ Matthew S. Lewis,¹ Awbrey N. Gilliam,¹ Abigail B. Ventura,¹ Daniel Malouli,¹ Guangwu Xu,¹ Rebecca Richards,¹ Nathan Whizin,¹ Jason S. Reed,¹ Katherine B. Hammond,¹ Miranda Fischer,¹ John M. Turner,¹ Alfred W. Legasse,¹ Michael K. Axthelm,¹ Paul T. Edlefsen,² Jay A. Nelson,¹ Jeffrey D. Lifson,³ Klaus Früh,¹ Louis J. Picker^{1*}

CD8⁺ T cell responses focus on a small fraction of pathogen- or vaccine-encoded peptides, and for some pathogens, these restricted recognition hierarchies limit the effectiveness of antipathogen immunity. We found that simian immunodeficiency virus (SIV) protein-expressing rhesus cytomegalovirus (RhCMV) vectors elicit SIV-specific CD8⁺ T cells that recognize unusual, diverse, and highly promiscuous epitopes, including dominant responses to epitopes restricted by class II major histocompatibility complex (MHC) molecules. Induction of canonical SIV epitope-specific CD8⁺ T cell responses is suppressed by the RhCMV-encoded *Rh189* gene (corresponding to human *CMV US11*), and the promiscuous MHC class I- and class II-restricted CD8⁺ T cell responses occur only in the absence of the *Rh157.5*, *Rh157.4*, and *Rh157.6* (human *CMV UL128*, *UL130*, and *UL131*) genes. Thus, CMV vectors can be genetically programmed to achieve distinct patterns of CD8⁺ T cell epitope recognition.

CD8⁺ T cells detect intracellular pathogens by T cell receptor (TCR)-mediated recognition of short pathogen-derived peptides selected and transported to the cell surface by MHC class I (MHC-I) proteins and an intricate system of intracellular peptide sampling and transport (*1*). Although pathogens can potentially generate many thousands of different peptides of the appropriate length for CD8⁺ T cell recognition, requirements for proteolytic processing, peptide transport, binding to available MHC-I allomorphs, and TCR repertoire matching, as well as poorly understood immunoregulatory mechanisms, winnow down these candidates to a relative handful of peptide epitopes that actually serve as targets for the CD8⁺ T cells that constitute antipathogen effector and memory responses (*2–4*). Remarkably, despite the complexity of the process, pathogen-specific CD8⁺ T cell responses mounted by individuals with shared MHC-I alleles tend to recognize an overlapping set of immunoprevalent epitopes (*2, 3, 5*). For the vast majority of pathogens, CD8⁺ T cell responses targeting such immunoprevalent epitopes are able to both recognize pathogen-infected cells and mount effective antipathogen effector and memory responses.

However, this is not the case for agents with efficient immune evasion capabilities, such as

HIV and its simian counterpart SIV. The massive replication of these viruses, combined with their high rate of mutation and functional plasticity, allows escape from most CD8⁺ T cell responses (*5, 6*). Indeed, CD8⁺ T cell responses in the majority of individuals infected with these viruses fail to target epitopes containing conserved, functionally critical viral sequences, and do not effectively control viral replication (*7*). Although vaccination against these viruses can greatly augment the magnitude of CD8⁺ T cell responses after infection, these larger CD8⁺ T cell responses target many of the same immunoprevalent epitopes recognized during infection of unvaccinated individuals, and therefore are still subject to immune escape (*6, 8, 9*). Although the AIDS vaccine field has endeavored to develop strategies capable of eliciting HIV/SIV-specific CD8⁺ T cell responses targeting “vulnerable” epitopes across diverse MHC-I haplotypes (either by increasing the overall recognition breadth of these responses or by focusing these responses to conserved sequences), this effort has not, to date, yielded strategies capable of substantially modifying CD8⁺ T cell immunodominance hierarchies, nor achieved the goal of establishing protective CD8⁺ T cell responses in the majority of individuals.

We recently reported an HIV/AIDS vaccine strategy that uses SIV protein-encoding RhCMV as a persistent vector to generate and maintain SIV-specific effector memory T cell responses intended to intercept SIV infection prior to the viral amplification needed for efficient immune evasion (*6*). Although this approach was not designed to prevent acquisition of infection, it proved to be highly successful; about 50% of RhCMV/SIV vector-vaccinated rhesus macaques chal-

lenged with highly pathogenic SIV manifested immediate, stringent, and durable virologic control (*10*). During the course of these studies, we noticed that RhCMV/SIV vectors did not elicit the canonical CD8⁺ T cell responses restricted by the well-characterized *Mamu-A1*001:01* (*A*01*) MHC-I allele. This raised the questions of which CD8⁺ T cell epitopes were targeted by these effective responses and whether differential targeting might have contributed to efficacy.

Here, we show that delivery of SIV antigens to the immune system via strain 68-I-based RhCMV/SIV vectors fundamentally changes CD8⁺ T cell recognition. Relative to conventional SIVgag-specific CD8⁺ T cell responses, the SIVgag-specific CD8⁺ responses elicited by the RhCMV/gag vector are broader by a factor of 3, and they target entirely different epitopes including an abundance of “supertopes” (highly promiscuous epitopes) and dominant MHC class II (MHC-II)-restricted CD8⁺ T cell responses that are rarely, if ever, observed in CD8⁺ T cell responses to any other infectious agent. Moreover, we demonstrate that this atypical CD8⁺ T cell targeting is under the genetic control of CMV; hence, it should be possible to genetically manipulate a vaccine vector to achieve distinct patterns of CD8⁺ T cell epitope recognition.

Results

Distinct CD8⁺ T Cell Epitope Targeting with RhCMV/SIV Vectors

We previously demonstrated that in contrast to other CD8⁺ T cell response-inducing SIV vaccines (*6*), the protection associated with RhCMV/SIV vector vaccination does not correlate with expression of protective MHC-I alleles (*10, 11*). Moreover, among a group of eight *Mamu-A*01*⁺ macaques given RhCMV/gag and rev/tat/nef (rtm) vectors in these efficacy studies, none developed measurable frequencies of CD8⁺ T cells recognizing the normally immunodominant Gag_{181–189} (CM9) and Tat_{28–35} (SL8) epitopes, as measured by MHC-peptide tetramer analysis, although all four of these macaques that were subsequently administered adenovirus 5 (Ad5) vectors expressing SIVgag and SIVtat inserts did manifest robust responses to these canonical epitopes (*10*). To confirm this effect in *Mamu-A*01*⁺ macaques and to determine whether a lack of response to *Mamu-A*01*-restricted canonical epitopes in RhCMV/SIV vector-vaccinated macaques extends to canonical epitopes restricted by other MHC-I alleles (table S1), we used a flow cytometric intracellular cytokine staining (ICS) assay (*10, 12*) to analyze such canonical responses in macaques expressing one or more of the *Mamu-A*01* (*n* = 8), *Mamu-A1*002:01* (*A*02*; *n* = 4), *Mamu-B*008:01* (*B*08*; *n* = 2), and *Mamu-B*017:01* (*B*17*; *n* = 4) alleles and vaccinated with our standard strain 68-I RhCMV/gag, /rtm, /env, and /pol vectors (Fig. 1A and table S2). In all cases, RhCMV/SIV vector-vaccinated ma-

¹Vaccine and Gene Therapy Institute and Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR 97006, USA. ²Population Sciences and Computational Biology Programs, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA. ³AIDS and Cancer Virus Program, SAIC Frederick Inc., Frederick National Laboratory, Frederick, MD 21702, USA.

*Corresponding author. E-mail: picker@ohsu.edu

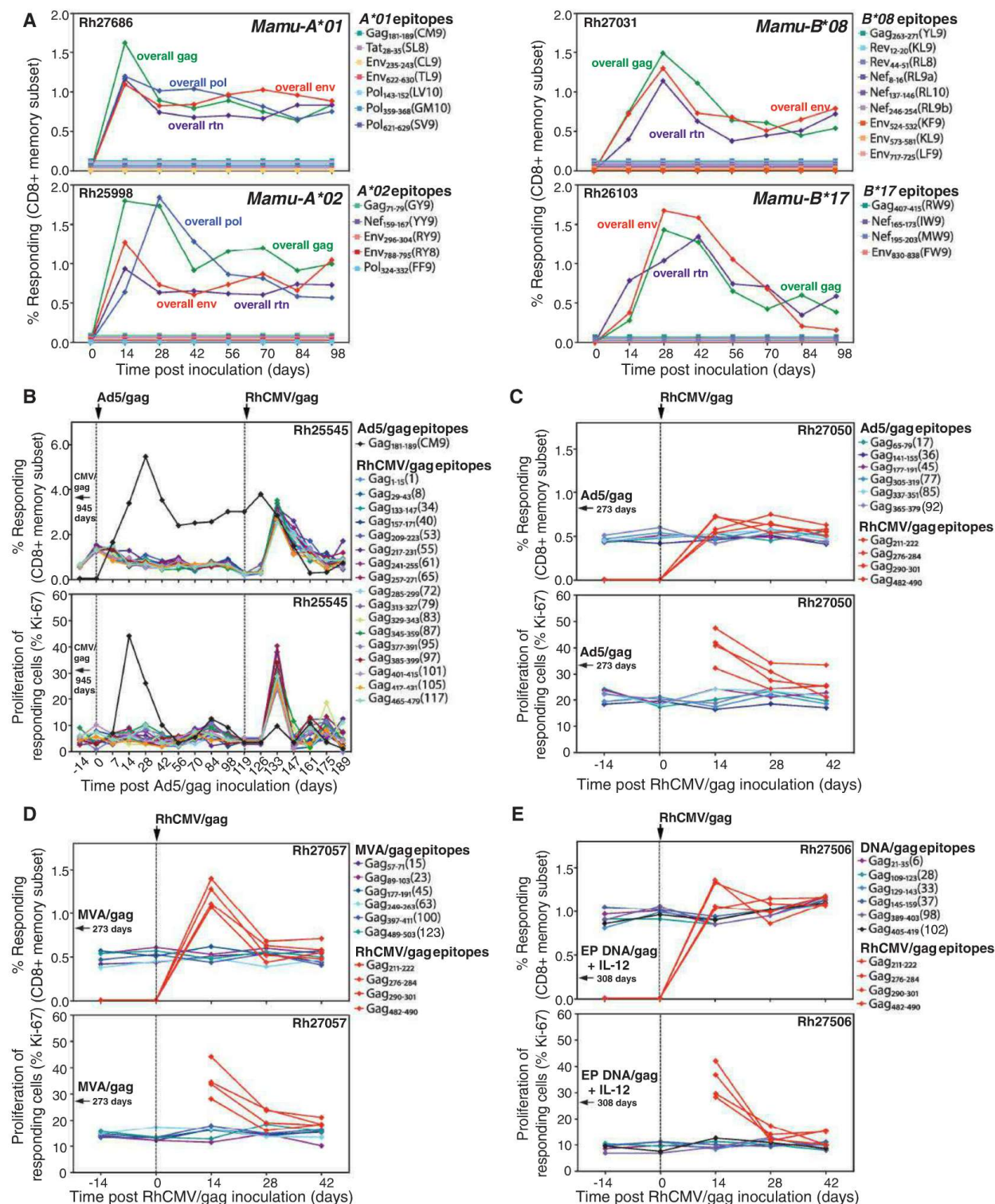


Fig. 1. Lack of epitope overlap between RhCMV vector-elicited and conventional SIV-specific CD8⁺ T cell responses. (A) Flow cytometric ICS was used to follow the development of overall SIV insert- and canonical epitope-specific CD8⁺ T cell responses (responder = TNF- α ⁺ and/or IFN- γ ⁺) in the blood of four macaques, each with a different well-characterized MHC-I allele (*Mamu-A*01*, *-A*02*, *-B*08*, and *-B*17*), after vaccination with strain 68-1 RhCMV/SIVgag, rev/tat/nef (rtn), env, and pol vectors. No response above background was observed for any canonical epitope. (B) The CD8⁺ T cell response to individual SIVgag 15mer peptides and the canonical Gag₁₈₁₋₁₈₉ (CM9) epitope was determined in a long-term strain 68-1 RhCMV/gag-vaccinated, *Mamu-A*01*⁺ macaque (Rh25545) by flow cytometric ICS. The frequency of these responses in

blood and the proliferating fraction among the responding cells (as measured by Ki-67 expression on TNF- α ⁺ and/or IFN- γ ⁺ events) were followed after boosting with Ad5/gag, and then 119 days later after revaccination with strain 68-1 RhCMV/gag. Gag₁₈₁₋₁₈₉ (CM9)-specific responses were not detected prior to administration of Ad5/gag. (C to E) The CD8⁺ T cell response to individual SIVgag 15mer peptides was determined in macaques vaccinated with Ad5/gag [(C), Rh27050], MVA/gag [(D), Rh27057], and electroporated DNA/gag + IL-12 [(E), Rh27506], and these responses were followed after vaccination with strain 68-1 RhCMV/gag as described in (B). These figures also show induction of 4 SIVgag 15mer-specific CD8⁺ T cell responses after RhCMV/gag vaccination that were not detectable in the pre-existing SIVgag-specific responses elicited by the conventional vaccines.

macaques manifested robust CD8⁺ T cell responses to the SIV protein containing the canonical epitopes, but these CD8⁺ T cell responses did not include a detectable response to any of the usually dominant or subdominant canonical epitopes restricted by any of these alleles.

These data indicate that the epitope hierarchies of CD8⁺ T cell responses elicited by RhCMV/SIV vectors are quite different from those elicited by SIV itself or by conventional vaccine vectors, but do not rule out low-level sensitization (below detection threshold) to canonical epitopes by RhCMV/SIV vectors or partial overlap between the epitopes targeted by RhCMV/SIV vector-elicited versus conventional vector-elicited responses. To explore these possibilities in outbred macaques, we used a heterologous prime/boost approach designed to determine the extent to which individual epitope-specific CD8⁺ T cell responses initially elicited by priming with RhCMV/SIV or conventional vectors could be subsequently boosted by the other vector type.

We first demonstrated the feasibility of this approach for RhCMV/gag by showing that SIVgag epitope-specific CD8⁺ T cell responses elicited by this vector are consistently boosted (as measured by a post-boost increase in the response frequency and proliferation of the epitope-specific CD8⁺ T cells) by a second administration of the same vector (fig. S1). We then determined the SIVgag epitope recognition profile of a *Mamu-A*01*⁺ macaque (Rh25545) primed with RhCMV/gag and the effect of a subsequent Ad5/gag boost on these individual epitope CD8⁺ T cell responses (Fig. 1B). We identified 17 distinct CD8⁺ T cell responses to individual SIVgag 15-amino acid oligomer (15mer) peptides after RhCMV/gag vaccination, but no response to the canonical *Mamu-A*01*-restricted Gag₁₈₁₋₁₈₉ (CM9) epitope. None of these 17 RhCMV/gag-elicited SIVgag epitope-specific CD8⁺ T cell responses were boosted by Ad5/gag, although all were boosted by subsequent administration of the RhCMV/gag vector. CD8⁺ T cells targeting the canonical *Mamu-A*01*-restricted Gag₁₈₁₋₁₈₉ (CM9) epitope were generated de novo by the Ad5/gag vaccine but were not boosted after the second administration of RhCMV/gag (indeed, these responses fell in frequency). Similar results were observed with a macaque (Rh27050) primed with Ad5/gag, another (Rh27057) primed with modified vaccinia Ankara (MVA)/gag, and two (Rh27506 and Rh27520) primed with DNA/gag + interleukin-12 (IL-12) (the latter administered by electroporation) and then boosted with RhCMV/gag (Fig. 1, C to E, and fig. S2), as well as in two SIV⁺ macaques (Rh25429 and Rh26557) on fully suppressive antiretroviral therapy that were administered RhCMV/SIV vectors (fig. S3). None of the SIVgag epitope-specific CD8⁺ T cell responses elicited by the priming with conventional vectors or by infection with SIV itself were boosted by RhCMV/gag, but administration of the latter vector did, in all cases, generate new CD8⁺ T cell responses to different (not previously

detected) SIVgag epitopes. Taken together, these data indicate that from the perspective of CD8⁺ T cell responses, the strain 68-1 RhCMV/SIV vectors and conventional vectors expressing the same SIV protein (as well as SIV itself) behave as if they present entirely different peptides to the immune system, manifesting nonoverlapping epitope recognition profiles.

Characteristics of Unconventional CD8⁺ T Cell Epitope Targeting

To comprehensively compare the epitope targeting profiles of SIVgag-specific CD8⁺ T cell responses elicited by strain 68-1 RhCMV/gag vectors versus conventional vectors and SIV itself, we used flow cytometric ICS to individually quantify CD8⁺ T cell responses to each of 125 consecutive 15mer peptides (with 11-amino acid overlap) comprising the entire SIVgag protein in 29 macaques: 14 vaccinated with RhCMV/gag vectors, 4 with electroporated DNA/gag + IL-12, 3 each with Ad5/gag and MVA/gag, and 5 with SIV infections controlled by conventional immune responses. Strikingly, peripheral blood CD8⁺ T cells from RhCMV/gag vector-vaccinated macaques responded to an average of 46 of these 125 SIVgag peptides, corresponding to a minimum of ~32 distinct epitopes (Fig. 2A). In contrast, SIV-infected controllers and macaques vaccinated with electroporated DNA/gag + IL-12, Ad5/gag, and MVA/gag responded to an average of 11 to 19 peptides, corresponding to an average of ~9 to 14 distinct epitopes. The breadth of the CD8⁺ T cell responses in the RhCMV/gag vector-vaccinated macaques was so great that many of SIVgag 15mer peptides were targeted by CD8⁺ T cells in most or even all of the 14 outbred macaques studied (Fig. 2A).

To determine whether this finding reflects promiscuous recognition of a single common epitope ("supertope") or is simply the result of CD8⁺ T cell recognition "hot spots" including multiple overlapping but different epitopes, we analyzed the CD8⁺ T cell response to a series of truncated peptides corresponding to seven of these commonly recognized 15mers in three macaques per response, so as to identify core epitopes in each macaque (Fig. 2B and fig. S4). Intriguingly, we identified two distinct response patterns with truncated peptides: type 1, in which response frequencies dropped abruptly with loss of a critical amino acid residue, typically yielding a 9mer core epitope (Gag₂₅₉₋₂₆₇, Gag₂₇₆₋₂₈₄, or Gag₄₈₂₋₄₉₀); and type 2, in which response frequencies only gradually declined as the optimal sequence was truncated, typically leaving a 12mer core epitope (Gag₄₁₋₅₂, Gag₂₁₁₋₂₂₂, Gag₂₉₀₋₃₀₁, or Gag₄₉₅₋₅₀₆). These truncation response patterns and core peptides were the same in all macaques studied for each response, and in all cases, the core peptides manifested superior stimulation (higher response frequencies) relative to the parent 15mer (Fig. 2C).

Taken together, these data strongly suggest that many of the SIVgag epitopes targeted by

CD8⁺ T cells in RhCMV/gag vector-vaccinated macaques are specific determinants that are commonly or even universally recognized across disparate MHC haplotypes. Indeed, a detectable CD8⁺ T cell response to the core (optimal) peptide for five of these truncated 15mers (including both type 1 and type 2 truncation patterns) was found in 100% of 42 RhCMV/gag-vaccinated, outbred macaques, and responses to six other peptides (two optimal peptides and four 15mers) were found in >60% of macaques (Fig. 2D, left panel). Notably, CD8⁺ T cells in 40 SIV-infected macaques rarely recognized these epitopes (Fig. 2D, right panel). Thus, relative to conventional SIVgag-specific CD8⁺ T cell responses, strain 68-1 RhCMV vector-elicited CD8⁺ T cell responses to SIVgag are broader by a factor of ~3 and are uniquely characterized by frequent targeting of broadly recognized supertopes.

MHC-I-restricted epitopes are typically 8 to 10 amino acids in length and have position-specific amino acids that engage binding pockets (anchor residues) so as to fit in a "closed-end" MHC-I binding groove (13); these characteristics are consistent with the type 1 truncation pattern described above. In contrast, the type 2 truncation pattern is more typical of MHC-II-restricted epitopes [which are typically longer (usually with a ≥12mer core), lack specific anchor residues, and are more tolerant of length heterogeneity (14, 15)]. This suggested that the CD8⁺ T cells recognizing type 2 SIVgag epitopes in the RhCMV/gag vector-vaccinated macaques might be MHC-II-restricted. In this regard, although MHC-II-restricted CD8⁺ T cell responses are clearly unusual, such responses have been reported in both mice (16–21) and humans (22–24), and it has been established that productive TCR signaling does not require specific CD4 or CD8 coreceptor engagement with MHC-II or MHC-I, respectively (25, 26). To investigate the possibility of MHC-II-restricted CD8⁺ T cell responses in our system, we assessed the ability of "blocking" monoclonal antibodies (mAbs) specific for MHC-I or MHC-II, and of the invariant chain-derived, MHC-II-specific binding peptide CLIP (27), to inhibit the type 1 epitope- and type 2 epitope-specific CD8⁺ T cell responses in the strain 68-1 RhCMV/gag-vaccinated macaques (Fig. 3A and fig. S5). Strikingly, inhibition of the seven supertope-specific CD8⁺ T cell responses by these reagents corresponded precisely to the type 1 versus type 2 truncation pattern, with CD8⁺ T cell recognition of the four type 2 epitopes (Gag₄₁₋₅₂, Gag₂₁₁₋₂₂₂, Gag₂₉₀₋₃₀₁, and Gag₄₉₅₋₅₀₆) blocked by the MHC-II mAb and CLIP, but not the MHC-I mAb, and the reverse for CD8⁺ T cell recognition of the three type 1 epitopes (Gag₂₅₉₋₂₆₇, Gag₂₇₆₋₂₈₄, and Gag₄₈₂₋₄₉₀). We then comprehensively characterized the epitope-specific responses mapped in Fig. 2A with respect to MHC-I versus MHC-II blockade (Fig. 3, B and C). All CD8⁺ T cell responses in the SIV-infected macaques and the macaques vaccinated with the conventional vaccines were blocked only by the MHC-I mAb,

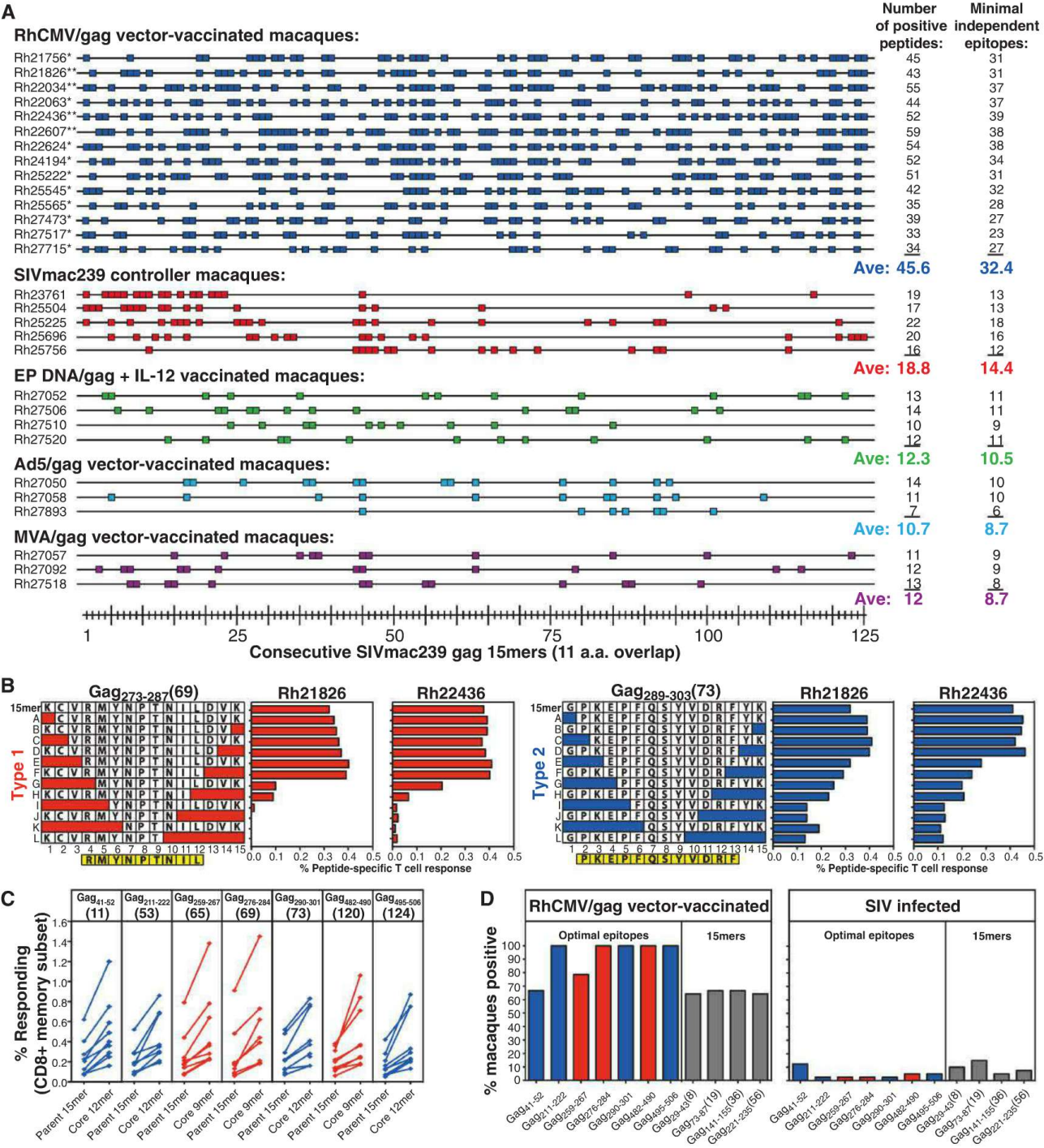


Fig. 2. RhCMV vector-elicited and conventional SIVgag-specific CD8⁺ T cell responses differ in epitope breadth and promiscuity. (A) CD8⁺ T cell responses to SIVgag were epitope-mapped using flow cytometric ICS to detect recognition of 125 consecutive 15mer gag peptides (with an 11-amino acid overlap) in macaques vaccinated with strain 68-1 RhCMV/gag vectors [*BAC-derived RhCMV/gag; **non-BAC-derived RhCMV/gag(L); *n* = 14], electroporated DNA/gag + IL-12 vectors (*n* = 4), Ad5/gag vectors (*n* = 3), and MVA/gag vectors (*n* = 3) and in SIV⁺ macaques with controlled infection (*n* = 5). Peptides resulting in above-background CD8⁺ T cell responses are indicated by a colored box, with the total number of these positive responses and the minimal number of independent epitopes potentially contained within these reactive peptides in each macaque designated at right. *P* < 0.0001, epitope breadth of RhCMV/gag-vaccinated macaques compared to macaques pooled over the other groups, using two-tailed Wilcoxon rank sum tests. **(B)** The core epitopes of selected 15mer

peptides targeted by CD8⁺ T cells from strain 68-1 RhCMV/gag-vaccinated macaques were determined by flow cytometric ICS analysis of CD8⁺ T cell responses to the indicated truncated peptides. The figure shows representative examples of the two response patterns observed with truncated peptide sets: type 1 (red), with abrupt loss of peak responsiveness and a 9mer core epitope, and type 2 (blue), with gradual loss of peak responsiveness and a 12mer core epitope (see also fig. S4). **(C)** CD8⁺ T cell response frequencies to the parent 15mer peptides and the core peptides derived from these 15mers (type 1, red; type 2, blue) were compared by flow cytometric ICS in nine macaques for each response. **(D)** CD8⁺ T cell responses to selected SIVgag core epitopes (type 1, red; type 2, blue), as well as selected additional SIVgag 15mers (gray), were evaluated by flow cytometric ICS in 42 strain 68-1 RhCMV/gag vector-vaccinated macaques (left panel) and 40 SIV-infected macaques (right panel); percentages of macaques in each category with detectable responses to these peptides are shown.

whereas in the RhCMV/SIV vector-vaccinated macaques, the CD8⁺ T cell responses to the majority of the targeted 15mers (63%) were specifically blocked by the MHC-II inhibitors, leaving

a minority (35%) blocked only by the MHC-I mAb (with ~2% of responses indeterminate).

To confirm that the MHC-II-blocked CD8⁺ T cell responses were MHC-II-restricted (here defined as the epitope in question being recog-

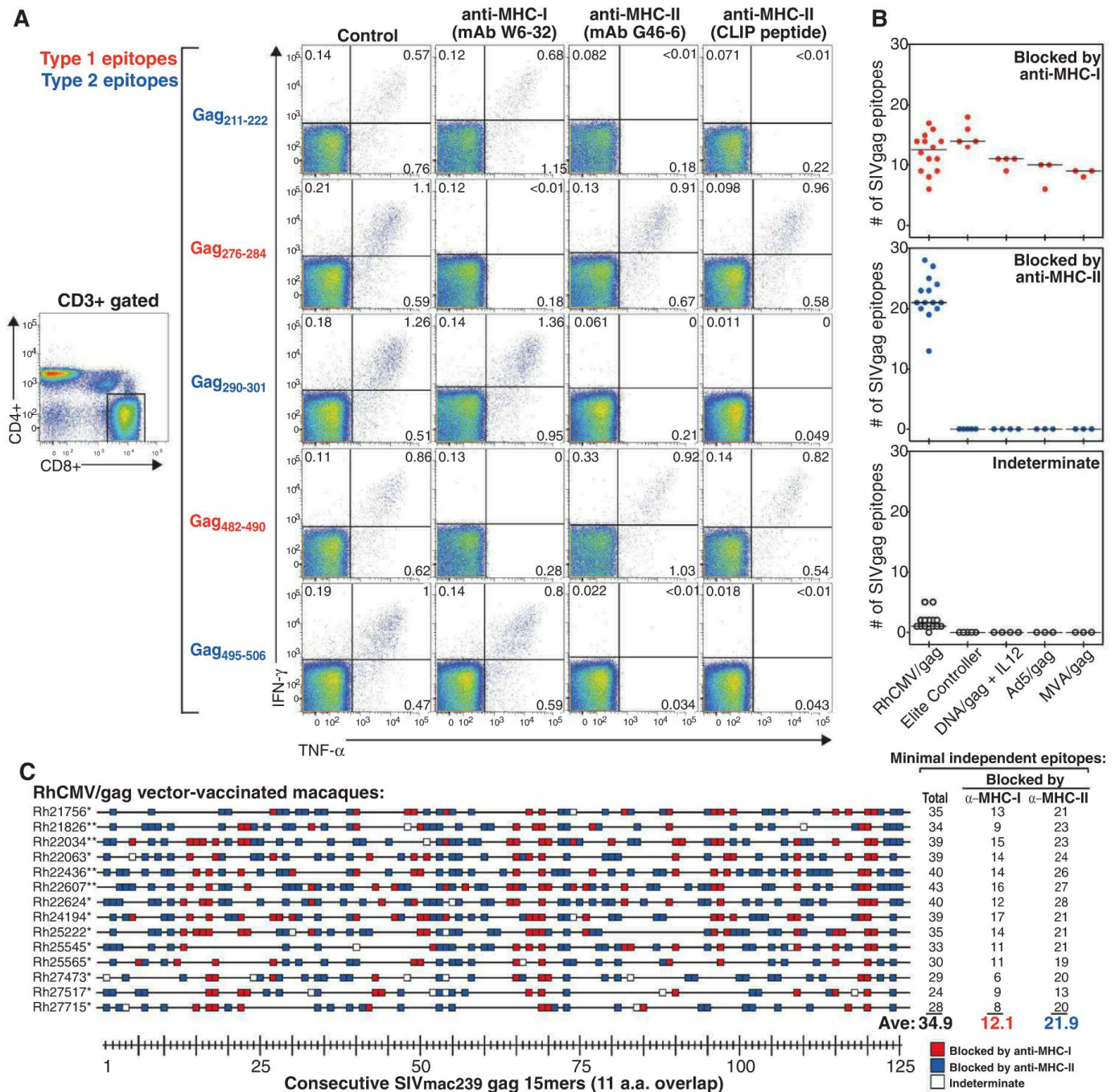


Fig. 3. Most RhCMV vector-elicited CD8⁺ T cell responses are inhibited by MHC-II blockade. (A) PBMCs from a representative strain 68-1 RhCMV/gag-vaccinated macaque (one of eight similarly analyzed; see fig. S5) were stimulated with the designated SIVgag core epitopes (type 1 versus type 2) in the presence of irrelevant isotype control mAbs (IgG₁, clone X40 + IgG_{2a}, clone X39; 10 μ g each), a MHC-I mAb (W6-32; 10 μ g), a MHC-II mAb (G46-6; 10 μ g), or the CLIP peptide (MHC-II-associated invariant chain, amino acids 89 to 100; 2 μ g). A typical CD4 versus CD8 expression profile, showing events gated on CD3⁺ small lymphocytes, is illustrated at the left, with the CD8 gate used to analyze the CD8⁺ T cell responses indicated. The IFN- γ versus TNF- α profiles (right panels) include only events collected through these gates, and thus reflect CD8^{hi}/CD4^{negative} T cells. (B and C) All the SIVgag 15mer peptide responses shown in Fig. 2A were

subjected to MHC-I mAb (W6-32) versus MHC-II mAb (G46-6) blockade and classified as being specifically inhibited (blocked) by MHC-I mAbs versus MHC-II mAbs, or indeterminate. (B) For each macaque, the average number of peptide-specific responses in each category is shown, classified by vaccine type. (C) The sensitivity of each SIVgag peptide response in 14 strain 68-1 RhCMV/gag-vaccinated macaques [*BAC-derived RhCMV/gag; **non-BAC-derived RhCMV/gag(L)] to blockade by MHC-I mAbs (red boxes) versus MHC-II mAbs (blue boxes) is shown (open boxes indicate indeterminate), with the minimal number of independent epitopes in the MHC-I- and MHC-II-associated categories designated at right (note: the total number of independent epitopes increased in some macaques from that listed in Fig. 2A because of the resolution increase afforded by the MHC blocking data).

nized in the context of an MHC-II surface protein) and to investigate the basis of the promiscuity of these responses across MHC-disparate macaques, we constructed cell lines expressing single rhesus MHC-II allomorphs, focusing on MHC-II alleles expressed by four RhCMV/gag-vaccinated macaques with characterized SIVgag epitope recognition profiles (fig. S6). Flow cytometric ICS assays showed that pulsing of the MHC-II allomorph transfectants, but not the parental MHC-II negative cell line, with individual peptides resulted in robust CD8⁺ T cell stimulation of only those responses classified as MHC-II-associated by blocking experiments (Fig. 4A and table S3), and these responses could be blocked with the MHC-II mAb and CLIP peptide, but not with the MHC-I mAb (fig. S7).

Individual MHC-II allomorphs presented multiple peptides, and individual peptides were frequently presented by multiple MHC-II allomorphs (Fig. 4A and table S3). The ability of individual allomorphs to present multiple gag peptides helps explain the striking breadth of these MHC-II-restricted CD8⁺ T cell responses. The ability of multiple MHC-II allomorphs to present many of the same individual peptides suggests that the common recognition of these peptides by RhCMV/gag vector-elicited CD8⁺ T cells across MHC-disparate macaques (e.g., their supertope character) is likely due to all macaques expressing at least one effective MHC-II allomorph for each response.

As previously reported for MHC-II-restricted CD4⁺ T cell responses (28), we found that MHC-II-restricted, SIVgag-specific CD8⁺ T cells elicited by RhCMV/gag vectors can respond to their specific peptide epitope in the context of peptide-binding MHC-II allomorphs that are not expressed by the responding T cell donor (Fig. 4, A and B, and table S3); these results indicate that the TCRs of these T cells recognize the bound peptide alone or in combination with nonpolymorphic structures on the MHC-II molecule. The expression of an MHC-II allomorph capable of binding a given epitope-containing peptide by a given macaque did not always result in a response to that peptide in that macaque [table S3; note the lack of a CD8⁺ T cell response to Gag₇₃₋₈₇(19) in Rh22034, to Gag₁₄₁₋₁₅₅(36) in Rh21826 and Rh22607, and to Gag₃₇₇₋₃₉₁(95) in Rh21826, despite the expression of MHC-II allomorphs by these macaques that can present these epitopes in other macaques] despite the expression of MHC-II allomorphs that can present these epitopes in other macaques. Such findings suggest that TCR repertoire and/or immunoregulatory mechanisms also play a role in determining the targeting of RhCMV vector-elicited CD8⁺ T cells, and in particular, whether epitopes are targeted by all vaccinated macaques or only a subset.

Phenotype and Function of RhCMV/SIV Vector-Elicited CD8⁺ T Cell Responses

The unusual epitope specificity of the SIV-specific CD8⁺ T cells generated and maintained by

RhCMV/SIV vector vaccination raises the question of their functional potential, especially that of the unconventional MHC-II-restricted population that dominates these CD8⁺ T cell responses. First, in this regard, these supertope-specific CD8⁺ T cell responses are not an artifact of the high peptide concentrations used in standard ICS assays, because responses to the optimal peptides, both type 1 and type 2, can be demonstrated at peptide dilutions of 1:10⁵ and greater (Fig. 5A). Second, both type 1 and type 2 supertope-specific CD8⁺ T cell responses arise immediately after vaccination (Fig. 5B) and are coordinately distributed throughout the body in the pattern previously reported for RhCMV/SIV vector-vaccinated macaques (Fig. 5, C and D) (10). Third, as previously reported for RhCMV-specific CD8⁺ T cells and RhCMV/SIV vector-elicited, SIV-specific CD8⁺ T cells (10, 11), both type 1 and type 2 supertope-specific T cells manifest an identical phenotype indicative of dominant effector memory cell differentiation (CCR7⁻, CD28⁻) and an identical polyfunctional profile consistent with this effector-memory phenotype: high production of tumor necrosis factor (TNF), interferon- γ (IFN- γ), and macrophage inflammatory protein-1 β (MIP-1 β); high externalization (degranulation) of lysosomal-associated membrane protein-1 (LAMP-1; CD107); and low IL-2 production (Fig. 5, E and F). Because effector memory differentiation is thought to be antigen-driven, these data suggest that in vaccinated macaques, these SIV-specific CD8⁺ T cells receive equivalent *in vivo* exposure to type 1 and type 2 epitopes.

The most important question regarding the function of the RhCMV/SIV vector-elicited CD8⁺ T cells is the extent to which they can recognize naturally processed SIV antigen and, in particular, SIV-infected cells. To evaluate this question, we compared the ability of RhCMV/SIV vector-elicited, SIV-specific T cells and conventional SIV-specific T cells from SIV-infected elite controllers to respond to (i) SIV virions chemically inactivated with aldrithiol-2 (AT-2), and (ii) autologous CD4⁺ T cells infected with SIV-mac239. We then used MHC-I versus MHC-II blockade to determine the restriction of this recognition. Previous work has established that AT-2-inactivated SIV (AT-2 SIV) is taken up by professional antigen-presenting cells (APCs) via both MHC-I and MHC-II processing pathways, the former by a fusion-mediated delivery of SIV virion proteins to the cytosol (29). CD4⁺ and CD8⁺ T cells from both RhCMV/SIV vector-vaccinated macaques and elite controllers responded to AT-2 SIV at concentrations down to <100 pg/ml of p27^{CA} equivalent (Fig. 6, A to C). The AT-2 SIV-specific CD4⁺ and CD8⁺ T cell responses from the SIV⁺ elite controllers were, as expected, completely and specifically inhibited with MHC-II- and MHC-I-blocking reagents, respectively. The AT-2 SIV-specific CD4⁺ T cell responses from the RhCMV/SIV vector-vaccinated macaques were similarly inhibited by MHC-II blockade, whereas the AT-2 SIV-specific CD8⁺

T cell responses from these macaques were partially inhibited by both the MHC-I- and MHC-II-blocking reagents, with the MHC-II blocking being most prominent (Fig. 6C and fig. S8).

At low AT-2 SIV concentrations (<1.6 ng/ml of p27^{CA} equivalent), the MHC-I-restricted component of the RhCMV/SIV vector-elicited CD8⁺ T cell response (the response present after blocking with the MHC-II binding CLIP peptide; blue line in Fig. 6C) disappears entirely, leaving only the MHC-II-restricted component (the response present after blocking with the MHC-I mAb; red line in Fig. 6C), indicating that the RhCMV/SIV vector-generated, MHC-II-restricted CD8⁺ T cells are more sensitive to AT-2 SIV than their MHC-I-restricted counterparts in this assay. This is likely because for AT-2 SIV, the MHC-II antigen presentation pathway processes and presents the MHC-II-restricted epitopes more efficiently than the cytosolic MHC-I pathway and/or cross-presentation can process and present the MHC-I-restricted epitopes.

We next compared the ability of SIV-specific T cells from RhCMV/SIV vector-vaccinated macaques and SIV⁺ elite controllers to recognize purified, autologous, SIV-infected CD4⁺ T cells. We evaluated the response of CD4⁺ and CD8⁺ T cells in peripheral blood mononuclear cell (PBMC) preparations and the response of isolated CD8⁺ T cells, the latter to rule out the possibility of indirect presentation of released SIV antigens by professional APCs. CD4⁺ and CD8⁺ T cells from PBMCs and isolated CD8⁺ T cells from all tested macaques specifically recognized purified SIV-infected CD4⁺ T cells, with the isolated CD8⁺ T cells showing the most robust response (Fig. 6, D and E). In these assays, the CD4⁺ T cell response to SIV-infected cells was, as expected, completely inhibited by MHC-II-blocking reagents in all macaques, whereas the CD8⁺ T cell responses to SIV-infected cells showed the same dichotomy described above for AT-2 SIV responses (Fig. 6F and fig. S9). The recognition of SIV-infected cells by the CD8⁺ T cells from SIV⁺ elite controllers was completely MHC-I-dependent, whereas the ability of CD8⁺ T cells from RhCMV/SIV-vaccinated macaques to recognize SIV-infected cells was ~70% inhibited by MHC-II blockade, and ~30% inhibited by MHC-I blockade. This implies that in RhCMV/SIV vector-vaccinated macaques, ~70% of the SIV-specific CD8⁺ T cells capable of recognizing autologous SIV-infected cells are MHC-II-restricted versus 30% MHC-I-restricted, a 2:1 ratio that closely approximates the ratio of the number of MHC-II- versus MHC-I-restricted epitopes recognized by CD8⁺ T cells in RhCMV/gag vector-vaccinated macaques (Fig. 3B). Taken together, these data demonstrate that the unconventional epitopes recognized by strain 68-1 RhCMV/SIV vector-elicited CD8⁺ T cells can be processed and presented by autologous SIV-infected cells, and therefore that this processing and presentation is independent of CMV gene expression.

CMV Genes Control Targeting of CMV-Elicited CD8⁺ T Cell Responses

The data described above strongly suggest that CMV has evolved specific mechanisms to modulate CD8⁺ T cell priming, perhaps to redirect the response away from the internally processed

epitopes that make CMV-infected cells vulnerable to CD8⁺ T cell-mediated cytotoxicity. Potential candidates for mediating this effect are the *Rh182*, *Rh184*, *Rh185*, and *Rh189* genes (the RhCMV orthologs of the *US2*, *US3*, *US6*, and *US11* genes in human CMV) that down-regulate

MHC-I expression in CMV-infected cells, preventing their recognition by CMV-specific CD8⁺ T cells (12, 30–32) (figs. S10 and S11). It is possible that these MHC-I down-regulators might prevent direct priming of CD8⁺ T cells by CMV-infected cells and thereby modify CD8⁺ T cell

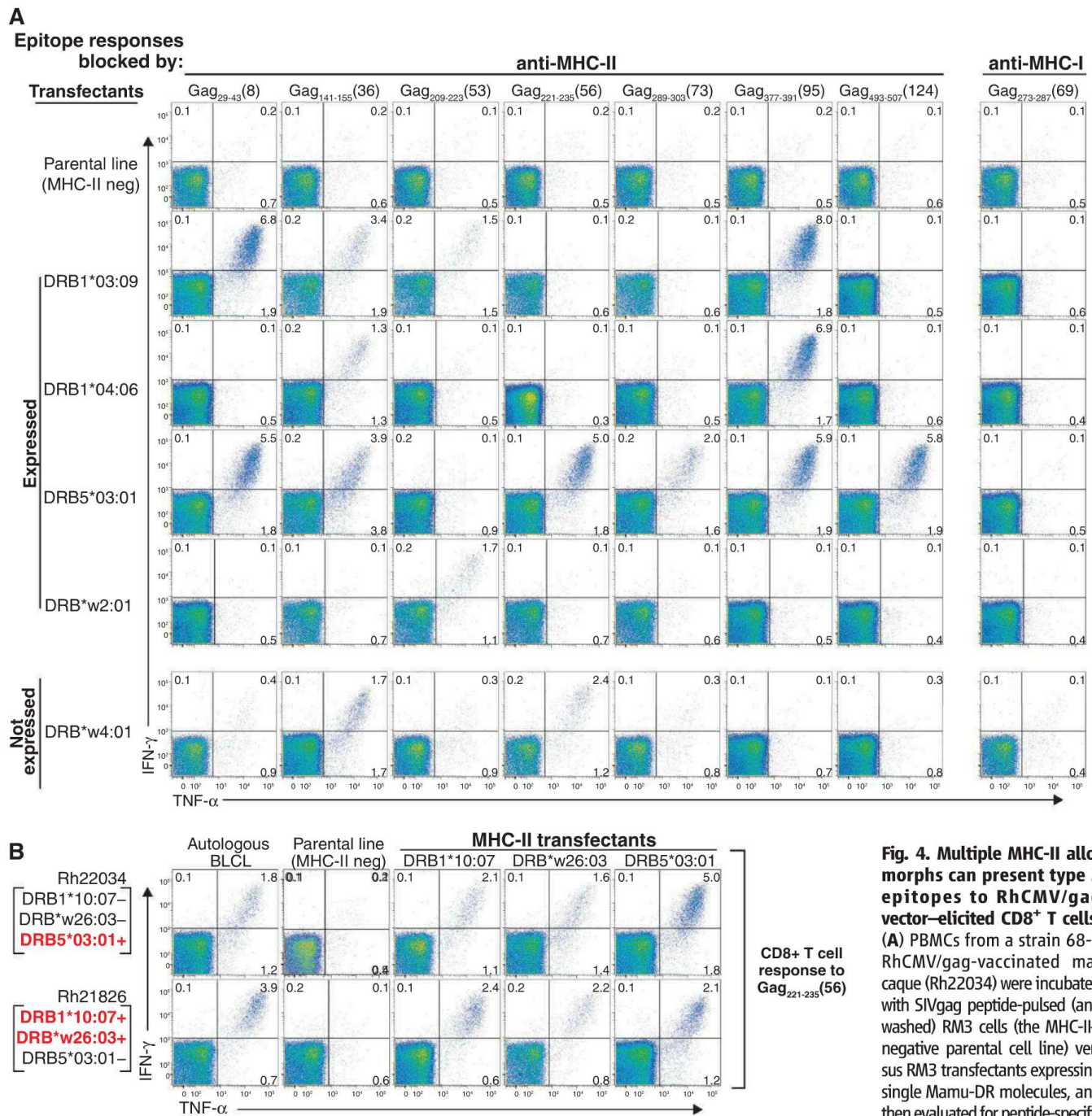


Fig. 4. Multiple MHC-II allomorphs can present type 2 epitopes to RhCMV/gag vector-elicited CD8⁺ T cells. (A) PBMCs from a strain 68-1 RhCMV/gag-vaccinated macaque (Rh22034) were incubated with SIVgag peptide-pulsed (and washed) RM3 cells (the MHC-II-negative parental cell line) versus RM3 transfectants expressing single Mamu-DR molecules, and then evaluated for peptide-specific CD8⁺ T cell recognition using flow cytometric ICS to detect induc-

tion of IFN- γ and/or TNF- α production (response frequencies are indicated in each quadrant). The Mamu-DR molecules tested included four that are expressed by Rh22034 (*DRB1*03:09*, *DRB1*04:06*, *DRB5*03:01*, and *DRB*w2:01*) and one that is not expressed (*DRB*w4:01*). The SIVgag 15mer peptides tested corresponded to known MHC-II-blocked CD8⁺ T cell epitopes (type 2) in this macaque, except for Gag₂₇₃₋₂₈₇ (15mer #69), which was MHC-I-blocked (type 1) and therefore used as a negative control. (B) Similar analysis of the presentation of the MHC-II-blocked (type 2) Gag₂₂₁₋₂₃₅ (15mer #56) peptide to CD8⁺ T cells from two strain 68-1 RhCMV/gag vector-vaccinated macaques (Rh22034 and Rh21836) by autologous B-lymphoblastoid cells, MHC-II null parental cells, and single MHC-II transfectants corresponding to Mamu-DRB alleles that are reciprocally expressed by these two macaques (expressed alleles denoted in red, non-expressed in black).

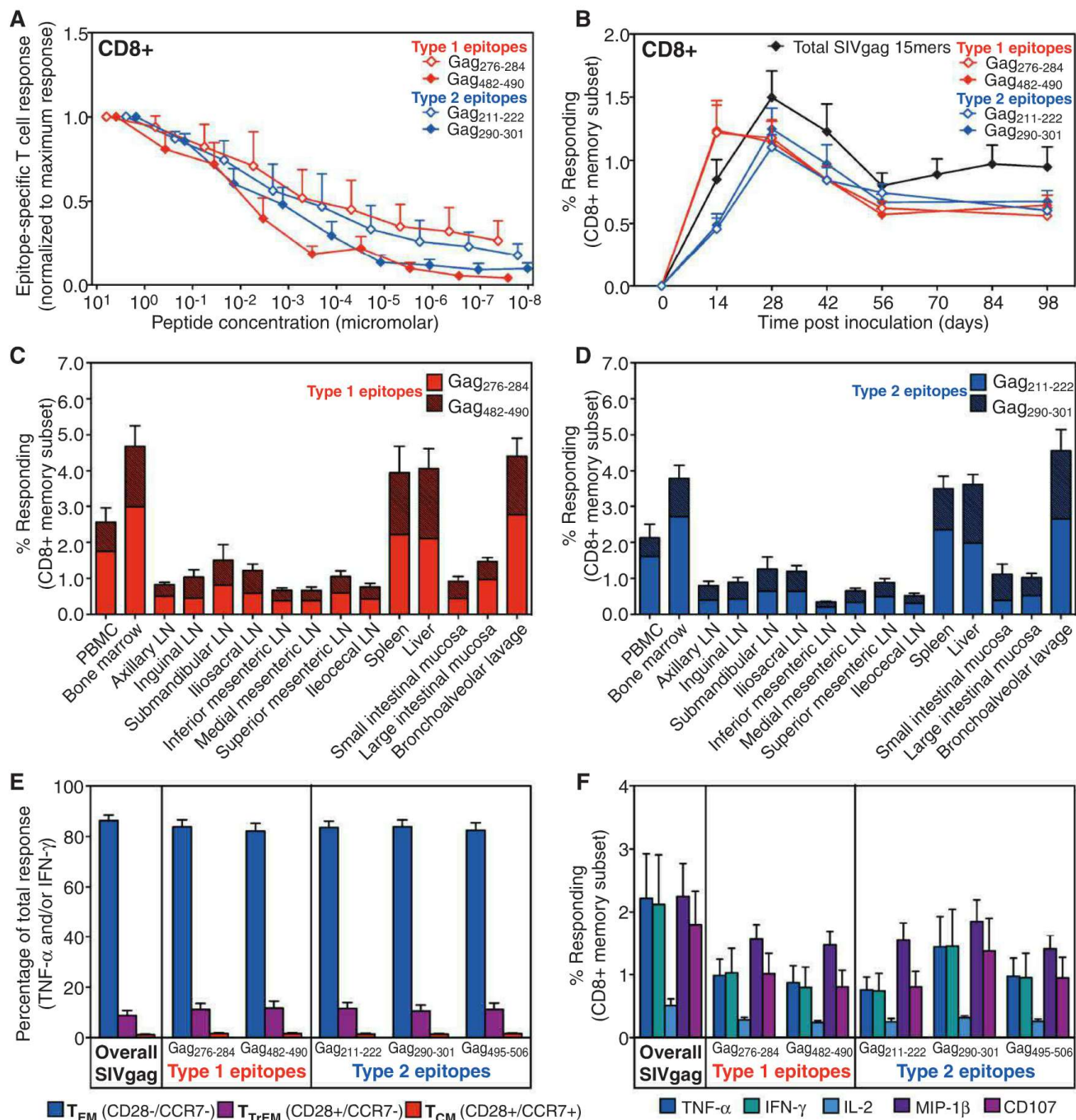


Fig. 5. RhCMV/gag vector-elicited CD8⁺ T cells show similar function regardless of MHC-I versus MHC-II restriction. (A) Serial log₁₀ dilutions of four core (optimal) SIVgag supertope peptides (two each MHC-I- and MHC-II-restricted) were used to stimulate PBMCs from strain 68-1 RhCMV/gag-vaccinated macaques ($n = 5$), and the response to each peptide dilution was determined by flow cytometric ICS. The frequency of responding CD8⁺ T cells (TNF-α⁺ and/or IFN-γ⁺) at each dilution was normalized to the maximal response at the initial peptide concentration. The figure shows the mean ± SEM of the normalized responses for each epitope. (B) Peripheral blood CD8⁺ T cell responses to total SIVgag 15mer mixes and to four core (optimal) SIVgag supertope peptides (two each MHC-I- and MHC-II-restricted) were quantified by flow cytometric ICS (TNF-α⁺ and/or IFN-γ⁺) after strain 68-1 RhCMV/gag vaccination (mean ± SEM; $n = 24$) to demonstrate the relative kinetics of induction of the MHC-I- versus MHC-II-restricted supertope responses. (C and D) CD8⁺ T cell responses to two MHC-I-restricted (C) and two MHC-II-restricted (D) core (optimal) SIVgag supertope peptides were

quantified by flow cytometric ICS (TNF-α⁺ and/or IFN-γ⁺) in mononuclear cell preparations from the indicated tissues at necropsy of strain 68-1 RhCMV/gag vector-vaccinated macaques (mean ± SEM; $n = 4$). (E) PBMCs from strain 68-1 RhCMV/gag-vaccinated macaques ($n = 14$) were stimulated with total SIVgag 15mer mixes or the MHC-I- versus MHC-II-restricted core (optimal) SIVgag supertope peptides shown and the expression of CD28 versus CCR7 was determined on the responding cells (TNF-α⁺ and/or IFN-γ⁺) by flow cytometric ICS, allowing delineation of the mean (±SEM) proportion of the responding cells manifesting the designated central memory (T_{CM}), transitional effector memory (T_{TEM}), and effector memory (T_{EM}) phenotypes. (F) PBMCs from strain 68-1 RhCMV/gag-vaccinated macaques ($n = 14$) were stimulated with total SIVgag 15mer mixes or the MHC-I- versus MHC-II-restricted core (optimal) SIVgag supertope peptides shown, and the frequencies of cells within the CD8⁺ memory compartment producing each cytokine or showing CD107 externalization were determined. The figure shows the mean (±SEM) of these response frequencies after background subtraction.

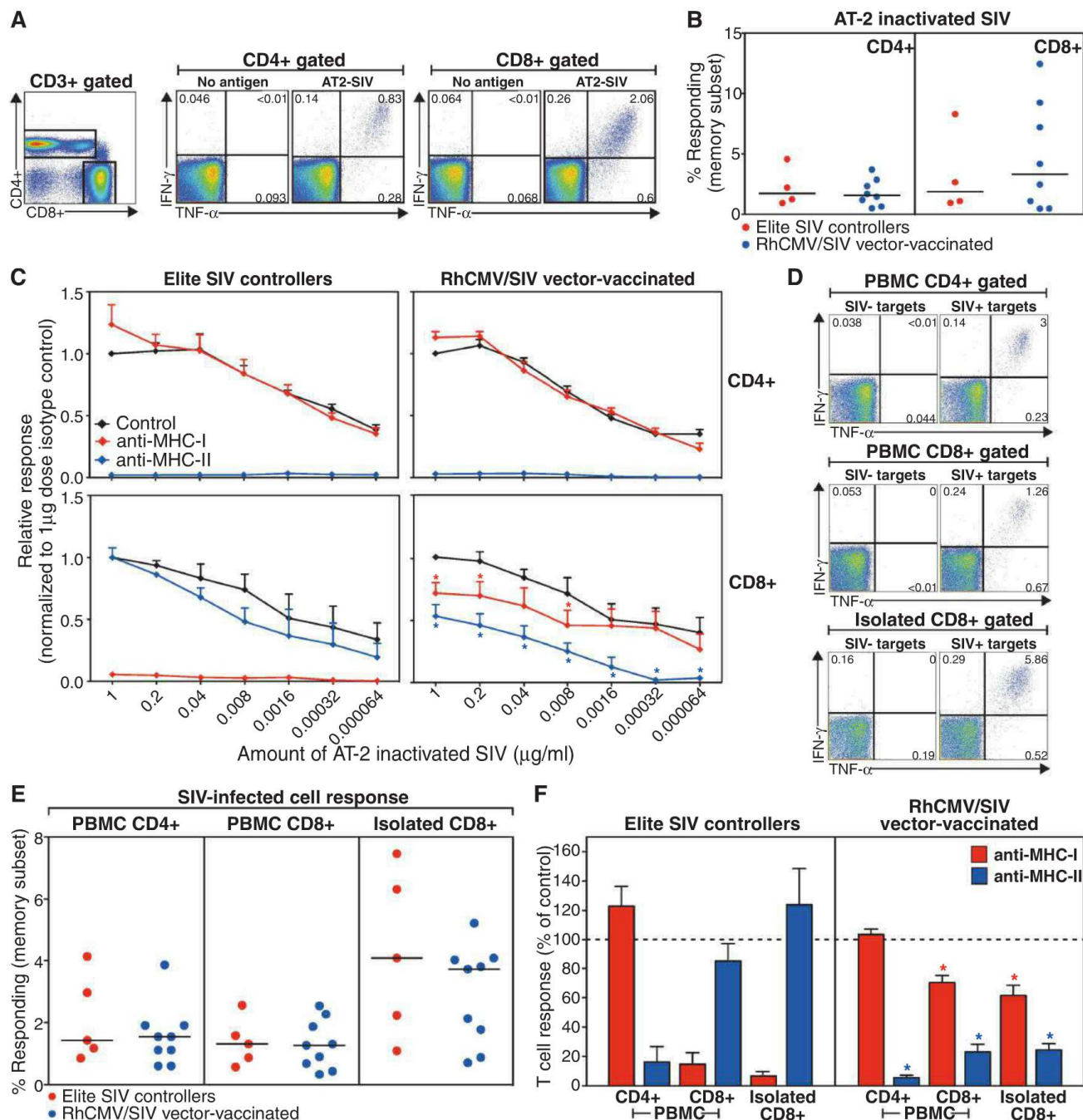


Fig. 6. RhCMV vector-elicited CD8⁺ T cells recognize SIV-infected cells via both MHC-I and MHC-II antigen presentation. (A) Representative flow cytometric profiles of CD4⁺ and CD8⁺ T cells in PBMCs from a macaque vaccinated with strain 68-1 RhCMV/SIV vectors responding to 1 μ g (p27^{CA} equivalent) of AT-2 SIV versus no antigen (quadrant response frequencies indicated). (B) Comparison of AT-2 SIV-specific response frequencies in the CD4⁺ or CD8⁺ memory compartment in the blood of SIV⁺ elite controllers ($n = 4$) versus macaques vaccinated with strain 68-1 RhCMV/SIV vectors ($n = 8$). (C) Serial fivefold dilutions of AT-2 SIV were used to stimulate PBMCs from the same strain 68-1 RhCMV/SIV-vaccinated and SIV⁺ elite controllers shown in (B), comparing the CD4⁺ and CD8⁺ T cell response frequencies in the presence of isotype control mAb versus MHC-I-blocking mAb W6-32 versus the MHC-II-blocking CLIP peptide (see Fig. 3). The response frequencies of each subset in each macaque were normalized to the unblocked response frequencies at the highest AT-2 SIV dose; means $\pm$ SEM of these normalized response frequencies are shown for each treatment and dose. Asterisks indicate the MHC-I- or MHC-II-blocked CD8⁺ T cell responses, as fractions of the isotype responses at

the same dilution, that are significantly different ($P < 0.05$) from 1.0 using a two-tailed Wilcoxon signed rank test (RhCMV/SIV vector group only; see fig. S8). (D) Representative flow cytometric profiles of CD4⁺ and CD8⁺ T cells in PBMCs and isolated CD8⁺ T cells from a macaque vaccinated with strain 68-1 RhCMV/SIV vectors responding to autologous SIV-infected CD4⁺ T cells (SIV⁺ targets) versus similarly processed and cultured CD4⁺ T cells that were not SIV-infected (SIV⁻ targets) using an effector-to-target ratio of 80:1. (E) Comparison of SIV-infected cell-specific response frequencies in the memory subsets of CD4⁺ and CD8⁺ T cells in PBMCs and isolated CD8⁺ T cells from SIV⁺ elite controllers ($n = 5$) versus macaques vaccinated with strain 68-1 RhCMV/SIV vectors ($n = 9$). (F) Sensitivity of the SIV⁺ cell-specific T cell responses shown in (E) to blocking with the MHC-I-blocking mAb W6-32 versus the MHC-II-blocking CLIP peptide. The response frequencies were normalized to the response frequencies in the isotype control; means $\pm$ SEM of these normalized response frequencies are shown for each treatment. Asterisks indicate the normalized MHC-I- or MHC-II-blocked CD8⁺ T cell responses that are significantly different ($P < 0.05$) from 100% using a two-tailed Wilcoxon signed rank test (RhCMV/SIV vector group only; see fig. S9).

epitope recognition hierarchies. Macaque fibroblasts infected with RhCMV/SIV vectors with the *Rh182–189* region deleted (Δ Rh182–189) are readily recognized by CD8⁺ effector T cells (fig. S11), and because of this susceptibility, these vectors fail to superinfect RhCMV⁺ macaques (12). However, these vectors can persistently infect CMV-naïve macaques and can elicit CD8⁺ T cell responses to SIV inserts in this setting (12). Strikingly, in sharp contrast to the RhCMV/SIV vectors with intact MHC-I down-regulation, Δ Rh182–189 RhCMV/SIV vectors elicit CD8⁺ T cells recognizing all tested *Mamu-A*01*–, *Mamu-A*02*–, and *Mamu-B*08*–restricted canonical epitopes (Fig. 7, A and B).

The targeting of canonical epitopes by the SIVgag-specific CD8⁺ T cells elicited by these Δ Rh182–189 (strain 68-1–derived) RhCMV vectors was in addition to, not in lieu of, the unconventional MHC-I– and MHC-II–restricted (supertope-directed) CD8⁺ T cell responses described above (Fig. 7C). It is also noteworthy that in contrast to conventional responses to these canonical epitopes, in which responses to certain epitopes are dominant and others subdominant (33, 34), the canonical epitope–targeted responses elicited by Δ Rh182–189 RhCMV/SIV vectors are all-inclusive, with each response very similar in magnitude (Fig. 7, A and B); this indicates that even when eliciting responses to conventional CD8⁺ T cell epitopes, these CMV-based vectors are still fundamentally different in their CD8⁺ T cell priming characteristics.

We next asked whether the appearance of canonical epitope recognition required complete abrogation of MHC-I down-regulation by construction and assessment of subregion deletant vectors (Δ Rh182–185 and Δ Rh186–189). These vectors only partially down-regulate MHC-I and partially prevent cytolytic T cell recognition (figs. S10 and S11), and, unlike the Δ Rh182–189 vectors, can superinfect CMV⁺ macaques, as evidenced by their ability to elicit SIV-specific T cell responses in this setting (Fig. 7D). If MHC-I down-regulation per se were the mechanism preventing canonical epitope recognition, one might predict that neither of these deletants would elicit such canonical epitope responses, or potentially, such responses would only be seen with the Δ Rh182–185 construct (lacking three of four MHC-I inhibitory genes—the *US2*, *US3*, and *US6* orthologs), which most closely resembles the full (Δ Rh182–189) deletant (fig. S11). However, this was not the case (Fig. 7D): Only the Δ Rh185–189 construct (lacking the *US11* ortholog) elicited CD8⁺ T cells against canonical epitopes (and did so for all tested epitopes restricted by four different MHC-I alleles), despite the very limited ability of the *Rh189* (*US11*) gene product to down-regulate MHC-I. The specific involvement of the *Rh189* (*US11*) gene in preventing canonical epitope recognition by RhCMV vector–elicited CD8⁺ T cells was confirmed by the development of such responses in macaques vaccinated with a RhCMV/SIV vector lacking

only the *Rh189* gene (Δ Rh189; Fig. 7E). Thus, *Rh189*, the ortholog of human CMV *US11*, specifically prevents priming of CD8⁺ T cells to canonical epitopes, almost certainly by a mechanism other than its known ability to target MHC-I molecules for degradation, because *Rh189* (*US11*) alone had only a very modest ability to both down-modulate MHC-I and inhibit T cell recognition of RhCMV-infected cells (figs. S10 and S11).

The presence of MHC-I– and MHC-II–restricted, supertope-directed CD8⁺ T cell responses in macaques administered Δ Rh182–189 RhCMV vectors indicates that other CMV-encoded mechanisms must be responsible for this unconventional CD8⁺ T cell targeting. To identify candidate CMV genes associated with, and potentially responsible for, this targeting, we first asked whether CD8⁺ T cell responses to CMV immediate early (IE) protein target unconventional epitopes (in particular, supertopes restricted by MHC-II), assessing both macaques naturally infected with wild-type RhCMV (colony circulating strains) and macaques vaccinated with strain 68-1 RhCMV/SIV vectors. Not surprisingly, macaques vaccinated with the strain 68-1 RhCMV/SIV vector demonstrated IE-specific CD8⁺ T cell responses with targeting characteristics identical to those of the SIVgag-specific CD8⁺ T cell responses in the same macaques: >30 distinct IE epitopes per macaque, including a majority of epitope-specific responses that were blocked with anti-MHC-II, and a minority blocked with anti-MHC-I. However, in striking contrast, the IE-specific CD8⁺ T cell responses in naturally RhCMV-infected macaques were much more narrowly targeted (~8 epitopes per macaque) and showed no evidence of MHC-II restriction or epitope promiscuity (Fig. 8, A to C, and fig. S12A), consistent with conventional immunodominance hierarchies. These findings likely account for why unconventionally targeted CMV-specific CD8⁺ T cell responses have not been reported in naturally exposed CMV⁺ macaques and humans (despite considerable analysis of these responses) and, more important, implicate genetic differences between the strain 68-1–based RhCMV vectors and wild-type RhCMV in the mechanism(s) responsible for generating the unconventionally targeted CD8⁺ T cell responses.

Most of the RhCMV/SIV vectors used in this study were derived from bacterial artificial chromosome (BAC)–cloned RhCMV strain 68-1, which was multiply passaged in fibroblast culture prior to its cloning. Relative to wild-type CMV, BAC-derived RhCMV has deletion or major defects in expression of the *Rh13.1*, *Rh61/Rh60*, *Rh157.5*, *Rh157.4*, and *Rh157.6* genes, corresponding, respectively, to the *RL13*, *UL36*, *UL128*, *UL130*, and *UL131* genes of human CMV (35, 36). To assess the role of these genes in the epitope targeting of CD8⁺ T cells primed by RhCMV/gag vectors, we inserted SIVgag into the previously constructed recombinant RhCMV68-1.2 in which functional ex-

pression of the *Rh61/Rh60*, *Rh157.4*, *Rh157.5*, and *Rh157.6* genes was restored (36). Strikingly, when macaques were inoculated with this repaired RhCMV/gag vector, the SIVgag-specific CD8⁺ T cell responses elicited did not include recognition of any of the previously defined MHC-I or MHC-II supertopes, were much more narrowly targeted than the response elicited by the unrepaired 68-1 strain vector, and were entirely MHC-I–associated (Fig. 8, D to F, and fig. S12B). Of the genes repaired in RhCMV68-1.2, *Rh61/Rh60* (*UL36*) could be ruled out as being responsible for this phenotype because this gene is intact in RhCMV/gagL, a non-BAC-derived precursor of recombinant RhCMV (12) that induces T cell responses similar to BAC-derived RhCMV (Fig. 2A). Therefore, we conclude that the RhCMV orthologs of *UL128*, *UL130*, and *UL131* preclude the priming of supertope-specific and MHC-II–restricted T cells. The proteins expressed by these genes associate with gH/gL to form a pentameric CMV virion surface complex involved in cell tropism [e.g., infection of non-fibroblasts (36)], suggesting a possible role for cell tropism in T cell priming. Notably, the SIVgag-specific response in *Mamu-A*01*⁺ macaques vaccinated with the repaired RhCMV/SIV vector did not develop responses to canonical Gag epitopes (Fig. 8D, bottom panel), which suggests that *US11* still precludes canonical epitope targeting in this setting. Thus, RhCMV encodes two independent mechanisms that inhibit the priming of CD8⁺ T cells targeting distinct epitope types: (i) *Rh189* (*US11*), which inhibits responses to canonical epitopes, and (ii) *UL128*, *UL130*, and *UL131* (*Rh157.5*, *Rh157.4*, and *Rh157.6*), which inhibit responses to highly promiscuous, unconventional epitopes, the majority of which are MHC-II–restricted.

Discussion

The CMVs are an extraordinarily successful, ancient family of viruses that have coevolved with their mammalian hosts. The persistent infections mediated by these viruses have a distinctive biology—a host-parasite relationship in which the virus elicits and maintains high-frequency, effector-differentiated T cell responses that stringently control its replication (preventing potentially life-threatening disease), but at the same time establishes immune evasion programs that prevent these responses from either clearing infection or interfering with viral transmission (11, 12, 37–39). Although, theoretically, viral evasion of CD8⁺ T cell responses might occur at both the afferent and effector phases of the response, the fact that CMV-specific CD8⁺ T cells constitute 10% or more of the circulating memory compartment in more than half of CMV⁺ individuals (38) would support the conclusion that the strategies used by CMV to evade these responses focus on protecting infected cells from CD8⁺ T cell effector function.

It has been proposed that viral-encoded MHC-I down-regulators might prevent direct

presentation and thereby inhibit or change the quality of CD8⁺ T cell priming; however, in the systems studied to date, the effect of these genes on CD8⁺ T cell priming has been minor at best (2, 31, 40–43), consistent with the major contribution of these genes being inhibition of CD8⁺

effector T cell recognition of virally infected cells. Indeed, the process of cross-presentation—the ability of noninfected professional APCs to initiate CD8⁺ T cell priming—would seem to be the host's evolutionary response to viral strategies aimed at inhibiting or substantially modifying

CD8⁺ T cell priming, unless they can act at a distance from infected cells (31). However, our data indicate that RhCMV can fundamentally manipulate CD8⁺ T cell priming with a surprising sophistication and stringency. Rather than preventing CD8⁺ T cell priming overall, RhCMV

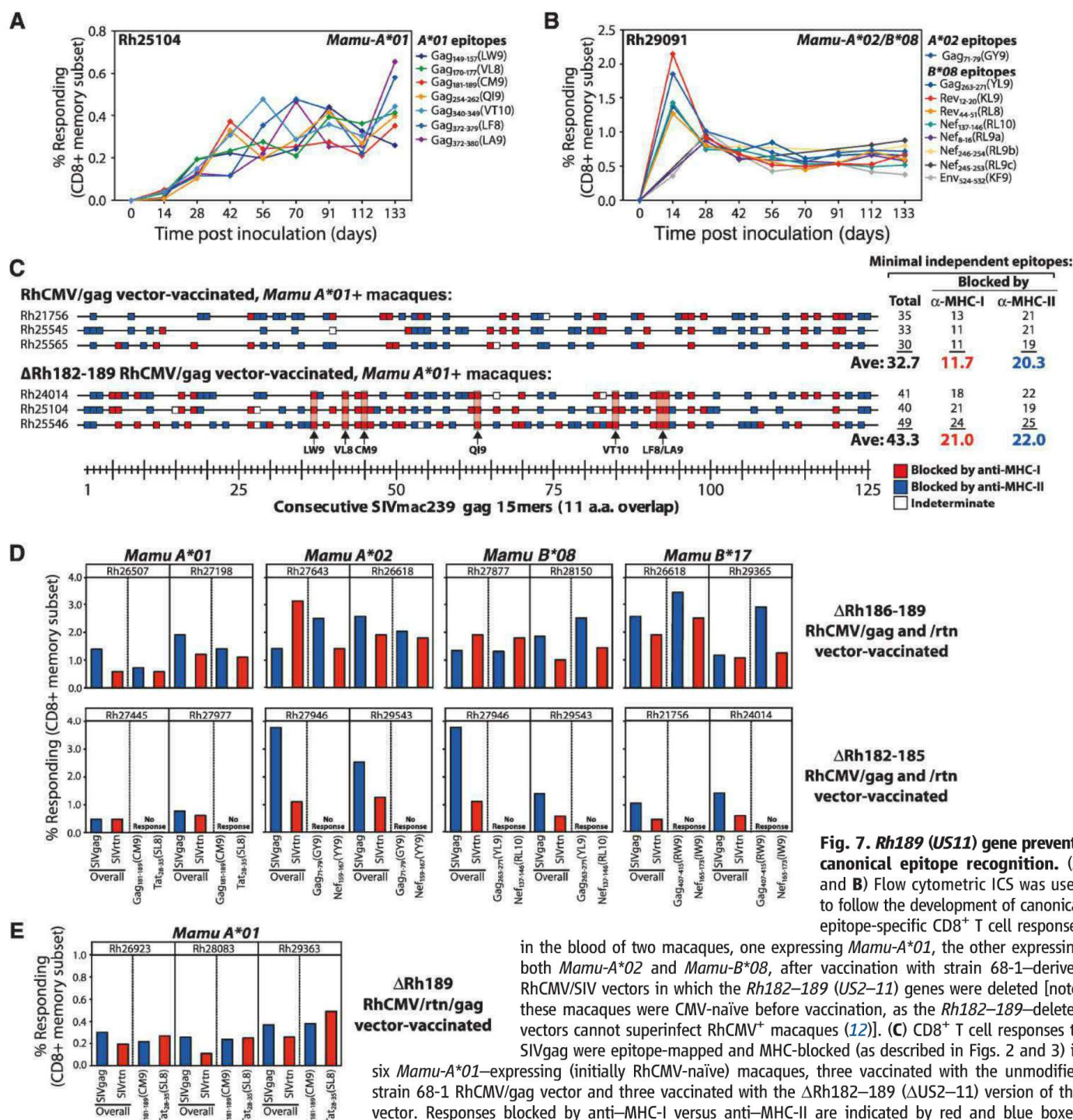


Fig. 7. *Rh189 (US11)* gene prevents canonical epitope recognition. (A and B) Flow cytometric ICS was used to follow the development of canonical epitope-specific CD8⁺ T cell responses

peptide mixes and canonical SIVgag, SIVnef, and SIVtat epitopes are shown for (initially RhCMV⁺) macaques expressing the designated MHC-I alleles and vaccinated with SIVgag- and SIVrtm-expressing strain 68-1 RhCMV vectors lacking either *Rh186–189 (US8–11)* or *Rh182–186 (US2–6)*. (E) The peak acute-phase CD8⁺ T cell responses in blood to whole SIVgag and SIVrtm peptide mixes and canonical SIVgag and SIVtat epitopes are shown for three (initially RhCMV⁺) macaques expressing *Mamu-A*01* and vaccinated with an SIVgag- and SIVrtm-expressing strain 68-1 RhCMV vector lacking only *Rh189 (US11)*.

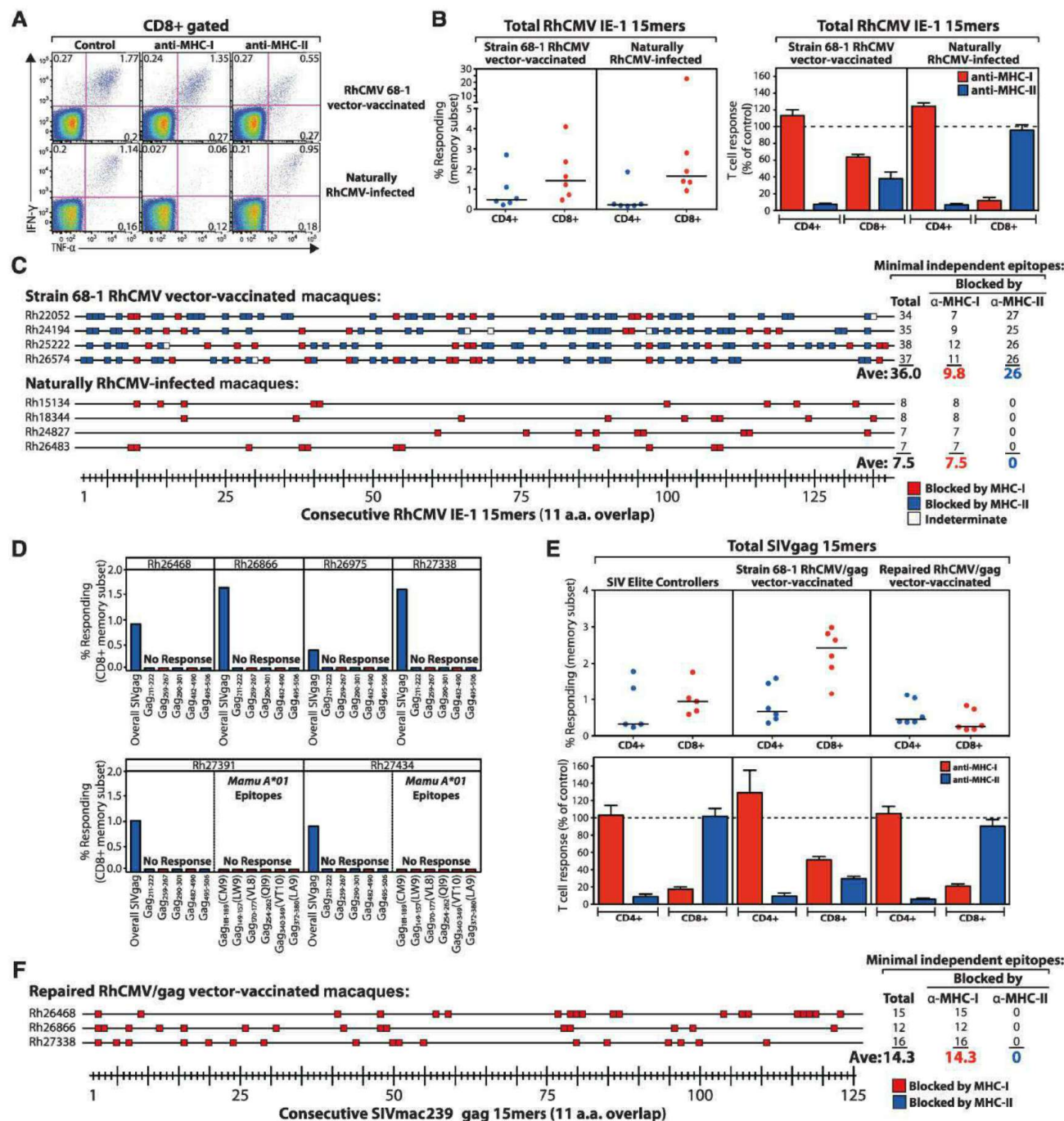


Fig. 8. Unconventional CD8⁺ T cell targeting is restricted to responses elicited by fibroblast-adapted RhCMV lacking UL128-131 ortholog expression. (A) Representative flow cytometric profiles of CD8⁺ T cells in PBMCs from an unvaccinated, naturally RhCMV-infected macaque (colony circulating strain) versus a strain 68-1 RhCMV/SIV vector-vaccinated macaque responding to consecutive 15mer peptides (11-amino acid overlap) comprising the RhCMV IE-1 protein in the presence of isotype control versus blocking MHC-I versus blocking MHC-II mAbs. (B) Comparison of the frequency (left panel) and sensitivity to blockade with MHC-I versus MHC-II mAbs (right panel; mean $\pm$ SEM) of IE-1-specific CD4⁺ and CD8⁺ T cells from naturally RhCMV-infected versus strain 68-1 RhCMV/SIV vector-vaccinated macaques (n = 6 per group; see fig. S12A). (C) CD8⁺ T cell responses to RhCMV IE-1 in naturally RhCMV-infected and strain 68-1 RhCMV/SIV vector-vaccinated macaques (n = 4 each) were epitope-mapped to determine recognition of 137 consecutive 15mer IE-1 peptides, and then the MHC association of each response was classified by sensitivity to blockade with MHC-I versus MHC-II mAbs. (D) The peak acute-

phase CD8⁺ T cell response frequencies in blood to the whole SIVgag 15mer mix, each of the five universal RhCMV/gag vector-associated supertopes, and in the two *Mamu A*01*⁺ macaques (Rh27391 and Rh27434), each of the indicated canonical SIVgag epitopes restricted by this allele, are shown in six macaques vaccinated with a strain 68-1 RhCMV/gag vector in which expression of RhCMV orthologs of HCMV UL128-131 genes (*Rh157.5*, *Rh157.4*, and *Rh157.6*) has been restored. (E) Comparison of the frequency (top panel) and sensitivity to blockade with MHC-I versus MHC-II mAbs (bottom panel; mean $\pm$ SEM) of SIVgag-specific CD4⁺ and CD8⁺ T cells from SIV⁺ elite controllers versus macaques vaccinated with the original strain 68-1 RhCMV/SIV vector versus macaques vaccinated with the *Rh157.4-6* (*UL128-131*)-repaired RhCMV/gag vector (n = 6 per group; see fig. S12B). (F) CD8⁺ T cell responses to SIVgag in three macaques vaccinated with the *Rh157.4-6* (*UL128-131*)-repaired RhCMV/gag vector were epitope-mapped and then the MHC association of each response was classified by sensitivity to blockade with MHC-I versus MHC-II mAbs (compare to Fig. 3C).

specifically and completely prevents the development of CD8⁺ T cells capable of recognizing certain types of CMV-encoded epitopes, redirecting the targeting of elicited CD8⁺ T cell responses to distinct epitope sets. Our results indicate that CD8⁺ T cell responses to a specific antigen can differ not only according to the differentiation state, physiology, and functional potential of the responding cells, as described (44), but also according to differential targeting of nonoverlapping epitopes.

Of the two components of CMV-mediated manipulation of CD8⁺ T cell targeting identified—the *Rh189* (*US11*)-associated inhibition of responses to canonical epitopes, and the inhibition of diverse MHC-I- and MHC-II-restricted super-epitope recognition associated with *Rh157.5*, *Rh157.4*, and *Rh157.6* (*UL128*, *UL130*, and *UL131*) [henceforth *Rh157.4–6* (*UL128–131*)]—the former is easier to conceptualize. Canonical epitopes are pathogen-derived peptides that are efficiently processed and presented by the MHC-I-linked endogenous antigen presentation pathway (given their predominant recognition across vectors that deliver the targeted proteins to the cytosol), and thus are likely to represent the most efficient targets for CD8⁺ T cell-mediated cytotoxicity of virally infected cells. Although CMV can actively and efficiently down-regulate MHC-I in infected cells, this activity cannot protect against cytotoxic T cell responses recognizing efficiently processed epitopes that are present in the cytoplasm of newly infected cells before these MHC-I down-regulators are expressed, including IE proteins and tegument and other viral proteins brought in with the infecting virion (45, 46). By preventing CD8⁺ T cell priming to such epitopes, this potential vulnerability is avoided. The ability of *Rh189* (*US11*) to mediate this specific inhibition of canonical epitope priming does not appear to derive from its known ability to target MHC-I molecules for proteolysis in infected cells, as by itself, *Rh189* (*US11*) can exert only modest MHC-I down-regulation, which was insufficient to prevent T cell stimulation. Moreover, this function would not easily explain the complete lack of any canonical epitope priming, which implies that both direct and cross-presentation of these epitopes is prevented (the latter being independent of MHC-I expression in infected cells). In this regard, it should be noted that although mouse CMV can down-regulate MHC-I, it does not encode a *Rh189* (*US11*) ortholog (47), and mouse CMV has been shown to be capable of eliciting responses to canonical epitopes (albeit by cross-presentation) (40, 42), which suggests that the *Rh189* (*US11*)-mediated mechanism is specific to primate CMVs.

The effect of the *UL128–131* orthologs on CD8⁺ T cell priming by RhCMV is more difficult to conceptualize, because it involves the ability of these genes to suppress priming of an entirely novel set of CD8⁺ T cell responses characterized by their targeting of diverse, highly promiscuous epitopes, predominantly in the context of MHC-

II presentation. The inescapable implication of this observation is that an intrinsic mechanism in RhCMV infection generates these unconventional CD8⁺ T cell responses, and therefore prompted the evolutionary development of the *Rh157.4–6* (*UL128–131*)-mediated mechanism to suppress this generation. In both RhCMV and HCMV, these genes (*Rh157.4–6*; *UL128–131*) encode three components of an alternative entry receptor for nonfibroblasts (36, 48, 49), but determination of whether the effect of this gene region on CD8⁺ T cell priming is a consequence of this function, of other reported functions of these genes [e.g., chemokine activity by *UL128* (50)], or of a completely new mechanism will require further analysis, as will understanding of the mechanisms by which RhCMV vectors lacking these genes elicit these unprecedented responses. Almost certainly, these mechanisms will delineate a new pathway of antigen presentation, perhaps one initiated by viral infection of fibroblasts or related stromal cells.

Although the mechanistic origin of the paradigm-breaking CD8⁺ T cell responses elicited by these strain 68-1 RhCMV vectors remains unclear, their existence indicates that previous understanding of the biology of CD8⁺ T cell epitope targeting is incomplete. First, there is the issue of the restriction of CD8⁺ T cell responses by MHC-II rather than MHC-I proteins. The specificity, breadth, and immediate response kinetics of the MHC-II-restricted CD8⁺ T cells identified here indicate that thymic selection pathways in primates do not preclude development and peripheral export of naïve CD8⁺ T cells with the ability to specifically recognize varied, MHC-II-bound peptides. Although this might reflect accidental MHC-II cross-reactivity of TCR selected on MHC-I molecules, as has been suggested for previous observations of CD8⁺ T cell alloreactivity against MHC-II (17, 22, 23), this cross-reactivity cannot be a rare “happencance” occurrence, but rather, given the diversity of the MHC-II-restricted CD8⁺ T cell responses we observed, must be an intrinsic feature of a degenerate repertoire. In this regard, many of the MHC-II-restricted CD8⁺ T cell responses studied in detail here, like many CD4⁺ T cell responses (28), recognize specific peptide epitopes in the context of multiple MHC-II allomorphs, including allomorphs not expressed by the T cells; this indicates that allele-specific MHC determinants do not make a major contribution to the recognition specificity of these T cells, and implies an intrinsic element of cross-reactivity in the TCR mediating these responses.

The second paradigm-violating aspect of the strain 68-1 RhCMV/SIV vector-elicited CD8⁺ T cell responses is the promiscuity of both the MHC-II- and MHC-I-restricted epitope recognition. Although MHC-II-restricted CD4⁺ T cell responses are intrinsically broader and more promiscuous than conventional MHC-I-restricted CD8⁺ T responses (14, 15, 51, 52), including descriptions of universal epitopes (53), the number

of universal MHC-II-restricted supertopes found in the RhCMV/gag-elicited CD8⁺ T cell responses would be highly unusual for a CD4⁺ T cell response, and the identification of multiple universal or nearly universal MHC-I-restricted supertopes is, to our knowledge, unprecedented (54). For the MHC-II-restricted responses, the promiscuity appears to reflect the ability of multiple MHC-II allomorphs to bind and present these supertopes, such that all (or most) macaques have at least one MHC-II allomorph that can bind and present the peptide involved in each response. The same mechanism may also apply to the MHC-I-restricted supertopes, although with MHC-I, there is the additional possibility that some or all of these MHC-I supertopes are presented by invariant MHC-I molecules (55). It is clear that the processing and presentation of these epitopes is not restricted to RhCMV-derived antigen, nor is in any way dependent on modification of antigen presentation pathways by the RhCMV vector, as both MHC-I- and MHC-II-restricted RhCMV/SIV vector-elicited T cells were able to specifically recognize naturally processed SIV virions and autologous SIV-infected CD4⁺ T cells. Given that, as discussed above, the naïve macaque CD8⁺ T cell repertoire must include T cells with unusual specificities described here and that naturally processed SIV antigens lead to presentation of the determinants recognized by these T cells, we must conclude that the failure to identify these CD8⁺ T cell specificities in the vast majority of CD8⁺ T cell responses to infectious agents or vaccines reflects peripheral immunoregulatory mechanisms that normally preclude such responses and/or favor conventionally targeted responses—mechanisms that must be bypassed by priming with strain 68-1 RhCMV vectors.

Our findings reveal a heretofore unrecognized flexibility in CD8⁺ T cell recognition, definitively demonstrate that the established rules of epitope selection and immunodominance are not absolute, and define a completely new kind of CD8⁺ T cell response that has the potential to be more effective than “natural” responses for some pathogens—particularly those for which natural responses are ineffective, such as HIV/SIV. Until efficacy analysis is performed on *Rh157.4–6* (*UL128–131*)-repaired RhCMV/SIV vectors [with and without *Rh189* (*US11*) deletion], it is unclear whether the distinct targeting of strain 68-1 RhCMV/SIV vector-elicited CD8⁺ T cell response is specifically responsible for the striking efficacy of these vectors against mucosal challenge with highly pathogenic SIV (10), but these responses almost certainly participate in this protection, as no other SIV-specific CD8⁺ T cells are available in the >50% of macaques that were stringently protected by this strain 68-1 RhCMV/SIV vector-containing vaccine.

Moreover, there are good reasons to believe that the unconventional CD8⁺ T cell responses generated by *Rh157.4–6* (*UL128–131*)-deficient CMV vectors might have specific advantages for HIV/SIV and perhaps other pathogens. First,

there is the enormous breadth of these responses. In the strain 68-1 RhCMV/gag vector-vaccinated macaques studied here, the elicited SIVgag-specific CD8⁺ T cells recognized an average of 22 MHC-II- and 12 MHC-I-restricted, largely nonoverlapping core epitopes (comprising 12 and 9 amino acids, respectively). Even allowing for 10% linear overlap, the core epitopes of these SIVgag-specific CD8⁺ T cell responses would encompass ~335 of the 510 amino acids in the SIVgag sequence, corresponding to 66% coverage of the protein! If recapitulated in an HCMV/HIV vaccine, such breadth would greatly increase the chance that vaccine-elicited CD8⁺ T cells would robustly recognize the divergent sequences in circulating HIV strains, as well as the likelihood that these responses will recognize vulnerable, conserved epitopes.

Second, the protection provided by these unconventional, supertope-targeted CD8⁺ T cell responses will be independent of specific MHC alleles, and therefore equally applicable to all individuals in all populations, in contrast to vaccines eliciting conventional responses that preferentially protect individuals already predisposed to T cell-mediated viral control by virtue of expression of protective MHC alleles.

Third, the fact that the epitope targeting of these unconventional CD8⁺ T cell responses is entirely distinct from the “natural” CD8⁺ T cell responses generated by HIV itself has crucial implications for both therapeutic and prophylactic AIDS vaccine development. In the case of a therapeutic vaccine administered to HIV⁺ individuals on suppressive antiretroviral therapy (ART), the virus being targeted by the vaccine will in many, if not most, subjects have escaped any effective conventionally targeted CD8⁺ T cell responses prior to viral suppression (7). A vaccine that simply boosts these natural, conventionally targeted, HIV-specific T cell responses would likely not provide control of such escaped viruses after ART withdrawal, whereas the unconventionally targeted T cells elicited by a CMV/HIV vaccine would not be affected by this prior escape. Given that HIV escape mutations may be transmitted, and therefore can become intrinsic components of circulating virus sequences (56), the same consideration would extend to a prophylactic HIV/AIDS vaccine; the efficacy of the unconventionally targeted CMV/HIV vaccine-elicited CD8⁺ T cells will not be affected by escape from the conventional targeting of natural CD8⁺ T cell responses.

Finally, the MHC-II restriction of the majority of the unconventional responses elicited by *Rh157.4–6* (UL128–131)-deleted CMV vectors might provide unique advantages for protection against pathogens that, like HIV and *Mycobacterium tuberculosis*, infect MHC-II⁺ cells (macrophages, activated T cells, etc.), essentially bringing the robust effector capabilities of the CD8⁺ T cell lineage (and its freedom from infection by HIV) to bear on MHC-II-expressing target cells (an effector response that would be in addition to

that mediated by the CD8⁺ effector T cells recognizing MHC-I-restricted epitopes).

We conclude that RhCMV has an intrinsic ability to elicit CD8⁺ T cell responses to unconventional epitopes, distinct in quality and quantity from all infectious agents studied to date. Moreover, this virus can exert unprecedented control of its own recognition by CD8⁺ T cells, with wild-type strains using *Rh157.5*, *Rh157.4*, and *Rh157.6* (UL128, UL130, and UL131) expression to divert CD8⁺ T cell targeting away from these unconventional epitopes, and *Rh189* (US11) expression to redirect responses away from the canonical epitopes that likely constitute the most efficient targets for cytolysis. These observations strongly hint that the relationship of this ancient virus and the mammalian cellular immune system is more complicated and intertwined than previously appreciated, and suggest that better understanding of this relationship will offer new insights into fundamental immunologic mechanisms. Moreover, these findings have practical implications for vaccine development, namely the ability to construct different CMV vectors (with or without US11; with or without UL128–131) that specifically elicit CD8⁺ T cell responses with widely divergent patterns of epitope recognition, including responses that break conventional immunodominance hierarchies. CMV thus constitutes the first programmable viral vaccine platform that allows custom targeting of vaccine-elicited CD8⁺ T cells to whichever set of epitopes is most appropriate for the pathogen in question.

Materials and Methods

Rhesus Macaques

A total of 165 purpose-bred male or female juvenile rhesus macaques (*Macaca mulatta*) of Indian genetic background were used in this study, including 110 macaques vaccinated with strain 68-1 RhCMV/SIV vectors (wild-type or genetically modified, alone or subsequent to heterologous priming with conventional vaccines or virally suppressed SIV infection), 47 macaques with SIV infection alone (SIVmac239 or SIVmac251), and 8 unvaccinated macaques that were naturally infected with colony-circulating strains of RhCMV. All macaques were used with the approval of the Oregon National Primate Research Center Institutional Animal Care and Use Committee, under the standards of the NIH Guide for the Care and Use of Laboratory Animals. Macaques used in these experiments were free of cercopithecine herpesvirus 1, D-type simian retrovirus, and simian T-lymphotrophic virus type 1. MHC-I genotyping for the *Mamu-A*01*, *Mamu-A*02*, *Mamu-B*08*, and *Mamu-B*17* alleles was performed by sequence-specific priming polymerase chain reaction (PCR), as described (57). Selected macaques were DRB-genotyped by deep sequencing. Briefly, amplicons of the *Mamu-DRB* region were created via amplification of cDNA by PCR with high-fidelity Phusion polymerase (NEBiolabs) and a pair of universal MHC-DRB-

specific primers (5'-CGTATCGCCTCCCTCGCGC-CATCAG-MID-CTGGTCTGTCTGTCTCTCC; 5'-CTATGCGCCTTGCCAGCCCGCTCAG-MID-TGGAAGGTCCAGTCTCCATT) using the following thermocycling conditions: 98°C for 3 min, (98°C for 5 s, 60°C for 10 s, 72°C for 20 s) for 25 cycles, and 72°C for 5 min. The primary cDNA-PCR products were purified using Ampure XP magnetic beads (Beckman Coulter Genomics). Emulsion PCR using a Lib-A kit (Roche/454 Life Sciences), bead purification, and pyrosequencing procedures with the Roche/454 GS Junior instrument were carried out as per the manufacturer's instructions. Data analysis was performed using a Labkey database in conjunction with Geneious-Pro bioinformatics software (Biomatters Ltd.) for sequence assembly. Mononuclear cell preparations for immunologic assays were obtained from blood, bone marrow, bronchoalveolar lavage (BAL), lymph nodes, spleen, liver, bone marrow, and intestinal mucosa, as described (58, 59). Purified CD8⁺ T cells (>90% pure) were obtained from PBMCs using CD8 microbeads and LS columns (Miltenyi Biotec). Plasma viral loads of SIV⁺ macaques were determined by quantitative real-time reverse transcription PCR (RT-PCR) (60). SIV⁺ macaques were considered SIV controllers if the plasma viral loads were <2.0 × 10⁴ copies/ml, and elite controllers if the plasma viral loads were <3.0 × 10³ copies/ml.

RhCMV/SIV Vectors

The construction, characterization, and administration of strain 68-1-derived RhCMV/SIV have been described in detail (10–12). All recombinant viruses used in this study were derived from strain RhCMV 68-1 BAC except for RhCMV(gagL), which was generated by replacing green fluorescent protein (GFP) in RhCMV-EGFP with the SIVgag expression cassette by in vivo recombination in tissue culture (12). Unlike BAC-derived constructs, RhCMV gagL contains an intact open reading frame (ORF), Rh61/Rh60 (UL36), as described for RhCMV68-1 (35, 61). As a result of tissue culture adaptation, both BAC and non-BAC RhCMV 68-1 constructs contain a deletion of ORF 157.5 and most of ORF Rh157.4 encoding homologs of HCMV UL128 and UL130, respectively (62). In low-passaged RhCMV, these two ORFs are translated from the same polycistronic mRNA encompassing Rh157.6 (UL131) (36). In RhCMV68-1 this transcript is truncated and the poly-A site is deleted, which suggests that Rh157.6 (UL131) is not expressed even though its coding region is intact. To generate a vector with complete UL128-131 expression, we inserted the SIVgag expression cassette into Rh211 of RhCMV68-1.2, a recombinant virus in which Rh61/Rh60 (UL36), Rh157.4 (UL130), and Rh157.5 (UL128) had been repaired (36). Deletion mutants within the Rh182-189 genomic region, which includes gene products inhibiting MHC-I antigen presentation, are shown in fig. S10. ΔRh182-189 RhCMV/gag has been

described (12). Similarly, we created Δ Rh182-189 RhCMV/rtn and /env by replacing the genomic region encoding Rh182-189 [base pairs 193,161 to 199,823, using the BAC genome annotation by (35)] with the EF1 α SIVrev/tat/nef or gH SIV/env expression cassettes. The partial deletion mutants Δ Rh182-185 RhCMV/gag and /rtn were generated by replacing base pairs 193,161 and 196,305 with an expression cassette for SIVgag or SIVrev/tat/nef. The partial deletion mutants Δ Rh186-189 RhCMV/gag and rtn were constructed by replacing base pairs 196,593 to 199,823 with SIVgag or SIVrev/tat/nef expression cassette. To generate recombinant RhCMV that only lacks *Rh189* (*US11*), we replaced the *Rh189* coding region with that of SIVgag in RhCMVrtn. This vector thus expresses SIVgag under control of the *Rh189* promoter and SIVrtn (inserted into Rh211) under control of the EF1 α promoter (fig. S10). All of the recombinant viruses were characterized and confirmed by restriction digestion, and the antigen inserts including their flanking regions were sequence-verified. Expression of SIV antigens was verified by immunoblot. Additionally, adjacent gene expression was verified by RT-PCR.

Other Vaccines

The construction, characterization, and administration of the Ad5/gag vectors used in this study have been described (10). MVA/gag was constructed by insertion of codon-optimized, full-length SIVmac239 gag gene into the MVA shuttle vector, pLW44, under the control of MH5, an early/late vaccinia promoter, to generate the recombinant plasmid, pJV7. Flanking sequences within pLW44 directed insertion of the recombinant construct into the thymidine kinase locus by homologous recombination. Chicken embryonic fibroblast cells were transfected with pJV7 followed by infection with MVA strain 1974 to generate recombinant virus expressing SIVmac239 gag (SIVgag expression confirmed by Western blot). Recombinant virus was plaque-purified and amplified in large-scale culture. Viral stocks were purified over a 24 to 40% sucrose gradient followed by pelleting through a 36% sucrose cushion with the pellet then suspended in 1 mM Tris-Cl, pH 9.0. For MVA/gag vaccination, macaques were administered 10^8 plaque-forming units of this vector via intramuscular injection. The DNA/gag + IL-12 vaccines were provided by Inovio Pharmaceuticals. Briefly, codon-optimized, 5' and 3' halves of the full-length SIVmac239 gag were cloned into the pVAX backbone (Invitrogen) such that the SIVgag insert expression was controlled by the human CMV (HCMV) promoter/enhancer and the bovine growth hormone polyadenylation signal. The optimized rhesus macaque IL-12 adjuvant was constructed via modification of a previously used unoptimized version of macaque IL-12 (63). Modification included codon and RNA optimization of the p35 and p40 insert sequences only, which was carried out by GeneArt (Invitrogen). Macaques

were administered 1 mg of the two SIVgag constructs and 0.5 mg of IL-12 construct with the DNA being delivered into the quadriceps muscle followed by in vivo electroporation using a Celectra constant-current device (Inovio Pharmaceuticals Inc.) as described (64).

Antigens and Antigen-Presenting Cells

The synthesis of sequential 15-mer peptides (overlapping by 11 amino acids) comprising the SIVgag, rev, nef, tat, env, and pol proteins and RhCMV IE-1 protein as well as specific 9- to 14-mer peptides within these proteins, was performed by Intavis AG, using the SIVmac239 sequence (GenBank accession number M33262) (65) or the strain 68-1 RhCMV IE-1 sequence (GenBank accession number AY186194) (61). All peptides are identified by the position of their inclusive amino acids from the N terminus (e.g., Gag₃₃₋₃₉). Consecutive 15mers are also designated by their 15mer position starting from the N-terminal 15mer (e.g., Gag₁₋₁₅ is 15mer #1; Gag₄₋₁₉ is 15mer #2, etc.). Unless otherwise specified, these peptides were used in T cell assays at 2 μ g/ml (whether alone or in pan-protein mixes). Aldrithiol-2-inactivated SIV (AT-2-SIV; lot P4146, AIDS and Cancer Virus Program, Frederick National Laboratory, Frederick, MD) was produced as described (29). Autologous B-lymphoblastoid cell lines (BLCL) were generated by infecting rhesus PBMCs with *Herpesvirus papio* (66). Autologous SIV-infected target cells were produced by spinoculation of activated CD4⁺ T cells with sucrose-purified SIVmac239, followed by 4 days of culture and then purification with CD4 microbeads and LS columns (Miltenyi Biotec), as described (67). Infected cell preparations were >95% CD4⁺ T cells and >50% SIV-infected after enrichment and were used at an effector:target ratio of 80:1. Construction of single Mamu-DR allomorph transfectants was performed as described (68), except that Mamu-DR alleles were inserted into plasmid pCEP4 (Invitrogen) rather than pcDNC3.1. *Mamu-DR**01:05 was paired with *DRB1**10:07, *DRB1**04:06, *DRB1**03:09, *DRB5**03:01, *DRB**w2:01, and *DRB**w26:03; *Mamu-DR**01:021 was paired with *DRB**w4:01. Prior to MHC-II restriction assays, mRNA from these transfectants was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen), amplified by RT-PCR using a universal primer pair (5'-GACCTGATGGTGCTGAGC-3' and 5'-GCTGC-ACTGTGAAGCTCTC-3') that spanned the highly polymorphic β 1 region of *Mamu-DRB*, and its sequence was confirmed. MHC-II transfectants and BLCLs were pulsed with the Gag peptide of interest at a final concentration of 5 μ g/ml for 90 min (37°C), then washed twice with warm PBS and once with warm R10 to remove unbound peptide before being used to stimulate freshly isolated PBMCs at an effector:target ratio of 10:1.

T Cell Assays

SIV- and RhCMV-specific CD4⁺ and CD8⁺ T cell responses were measured in mononuclear

cell preparations from blood and tissues by flow cytometric ICS, as described in detail (10–12). Briefly, mononuclear cells or isolated CD8⁺ T cells were incubated with antigen (peptide, AT-2 SIV, peptide-pulsed BLCLs or MHC-II transfectants, or SIV-infected CD4⁺ T cells) and the costimulatory molecules CD28 and CD49d (BD Biosciences) for 1 hour, followed by addition of brefeldin A (Sigma-Aldrich) for an additional 8 hours. Costimulation without antigen served as a background control. The MHC association (MHC-I versus MHC-II) of a response was determined by preincubating isolated mononuclear cells or APCs for 1 hour at room temperature in the presence of MHC-I mAb (10 μ g/ml; clone W6-32) versus MHC-II mAb (HLA-DR; clone G46-6) or CLIP peptide (MHC-II-associated invariant chain, amino acids 89 to 100; 2 μ g/ml) before adding peptides or combining effector and target cells and incubating per the standard ICS assay. Stimulated cells were fixed, permeabilized, and stained as described (10–12), and flow cytometric analysis was performed on an LSR-II instrument (BD Biosciences). Analysis was done using FlowJo software (Tree Star). In all analyses, gating on the light scatter signature of small lymphocytes was followed by progressive gating on the CD3⁺ population and then the CD4⁺/CD8⁺ versus CD4⁺/CD8⁺ T cell subsets. Antigen-specific response frequencies for CD4⁺ or CD8⁺ T cell populations were routinely determined from intracellular expression of CD69 and either or both IFN- γ and TNF- α [in select experiments, responses were also characterized by intracellular CD69 and either IL-2 or MIP-1 β production or CD107 externalization (11)]. In other select experiments, Boolean gates of (CD69⁺/TNF- α ⁺ and/or CD69⁺/IFN- γ ⁺) were generated and expression of CD28 and CCR7 was determined on the gated (responding) CD8⁺ T cell population (11). Response frequencies were reported after background subtraction and memory correction, as described (11, 58). For epitope deconvolution experiments, stricter response criteria were used to prevent false positives. In these studies, a response to a given 15mer peptide was considered positive if the frequency of events clustered as CD69⁺, TNF- α ⁺, and IFN- γ ⁺ was $\geq 0.05\%$, with background <0.01% in at least two independent assays. The classification of individual peptide responses as MHC-I- versus MHC-II-associated was based on >90% inhibition of the response by either MHC-I or MHC-II blockade relative to the isotype control. Responses that did not meet these criteria were considered indeterminate. Minimal independent epitope numbers were estimated from the positive responses identified by testing of consecutive 15mer peptides by the following criteria: single positive peptide = 1 independent epitope; 2 adjacent positive peptides = 1 independent epitope; 3 adjacent positive peptides = 2 independent epitopes; 4 adjacent positive peptides = 2 independent epitopes; and 5 adjacent positive peptides = 3 independent epitopes. These estimations of the

minimal number of independent epitopes were initially conducted without the benefit of the MHC association data, but were then revised using the same criteria, applied independently for MHC-I- versus MHC-II-blocked responses.

Statistics

For comparisons of independent samples, we applied bivariate Mann-Whitney U tests (69), also known as Wilcoxon rank sum tests. For one-sample comparisons to a fixed null-hypothesized value (such as percentages compared to 100%), we applied one-sample Wilcoxon signed rank tests (69). All tests were conducted as two-tailed tests with a type I error rate of 5%. We used the R statistical computing language (70) for all statistical analyses.

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Supplementary Materials

www.sciencemag.org/content/340/6135/1237874/suppl/DC1
Figs. S1 to S12
Tables S1 to S3

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Zircon U-Pb Geochronology Links the End-Triassic Extinction with the Central Atlantic Magmatic Province

Terrence J. Blackburn,^{1*†} Paul E. Olsen,² Samuel A. Bowring,¹ Noah M. McLean,¹ Dennis V. Kent,^{2,3} John Puffer,⁴ Greg McHone,⁵ E. Troy Rasbury,⁶ Mohammed Et-Touhami⁷

The end-Triassic extinction is characterized by major losses in both terrestrial and marine diversity, setting the stage for dinosaurs to dominate Earth for the next 136 million years. Despite the approximate coincidence between this extinction and flood basalt volcanism, existing geochronologic dates have insufficient resolution to confirm eruptive rates required to induce major climate perturbations. Here, we present new zircon uranium-lead (U-Pb) geochronologic constraints on the age and duration of flood basalt volcanism within the Central Atlantic Magmatic Province. This chronology demonstrates synchronicity between the earliest volcanism and extinction, tests and corroborates the existing astrochronologic time scale, and shows that the release of magma and associated atmospheric flux occurred in four pulses over about 600,000 years, indicating expansive volcanism even as the biologic recovery was under way.

The approximate temporal coincidence between the five major extinction events over the past 542 million years and the eruption of large igneous provinces (LIPs) has led to speculation that environmental perturbations generated by the emplacement of large volumes of magma and associated outgassing over short periods of time triggered each global biologic crisis (1). Establishing an exact link between extinctions and LIP eruptions has proved difficult because of the geographic separation between LIP volcanic deposits and stratigraphic sequences preserving evidence of the extinction. In most cases, uncertainties on radioisotopic dates used to correlate between geographically separated study areas exceed the duration of both the extinction interval and LIP volcanism by an order of magnitude. This hinders evaluation of any relationship between magmatism and extinction and precludes accurate estimates of volcanic effusion rates, associated volatile release, and extinction mechanisms.

The end-Triassic extinction (ETE)—marked within early Mesozoic basins of eastern North America by a dramatic turnover in fossil pollen, spores (sporomorphs), and vertebrates (2)—is one of the largest Phanerozoic mass extinctions,

occurring just before the Triassic-Jurassic boundary (3, 4), and has long been thought to be associated with the eruptions of the Central Atlantic Magmatic Province (CAMP) (5, 6). CAMP is the most acrially extensive LIP on Earth, and with volume estimates between $2\text{--}3 \times 10^6 \text{ km}^3$, it ranks as one of the most voluminous (7) (Fig. 1). Remnants of CAMP are found on four continents and consist primarily of continental tholeiitic basalts emplaced as subaerial flows and intrusive bodies during rifting of the Pangean supercontinent and incipient formation of the Atlantic Ocean basin (Fig. 1). Estimates of the timing and duration of CAMP provided by astrochronology (8, 9) and $^{40}\text{Ar}/^{39}\text{Ar}$ geochronology (10, 11) differ by an order of magnitude, preventing high-precision tests of the relationship between LIP volcanism and mass extinction. Use of the astrochronologic

time-scale, though potentially precise, relies on (i) recognition of the influence of orbital cycles in the rock record and (ii) the ability to predict orbital durations in the deep geologic past, both of which have engendered doubts about the reliability of the technique. The lower precision of $^{40}\text{Ar}/^{39}\text{Ar}$ dates (fig. S1) prevents estimation of the volume of magma erupted over unit time, a critical factor for evaluating extinction mechanisms such as CO_2 -induced global warming (12, 13) ocean acidification (14, 15), or sulfur aerosol-induced “volcanic winters” (16).

U-Pb Geochronology of CAMP Flows and Intrusives

Here, we present zircon (ZrSiO_4) U-Pb geochronologic data for CAMP magmatism from seven sites in eastern North America and one in Morocco (Fig. 1), integrated with paleobiological, geochemical, and paleomagnetic data derived from sedimentary sequences interbedded with and intruded by the magmatic rocks. These data provide (i) a precise determination of the onset and duration of CAMP magmatism and (ii) a test of the reliability of the astronomical time scale in order to provide a high-precision age model for the CAMP-ETE interval (8, 9). Though U-Pb dating of zircon permits an order of magnitude improvement over previous $^{40}\text{Ar}/^{39}\text{Ar}$ studies (fig. S1), this preferred mineral for U-Pb dating is uncommon in basaltic rocks. This forced our sample collection to focus on finding isolated coarse-grained segregations within gabbroic intrusions and thick subaerial flows where incompatible element-enriched residual melts resulted in the crystallization of zircon (17) (fig. S2). The reported zircon U-Pb dates are ^{238}U - ^{206}Pb weighted mean dates with $\pm 2\sigma$ analytical uncertainties corrected for initial ^{230}Th disequilibrium (figs. S3 and S4) (18).

Stratigraphically constrained basalt flows, such as the North Mountain and Preakness basalts, provide the most straightforward means of directly

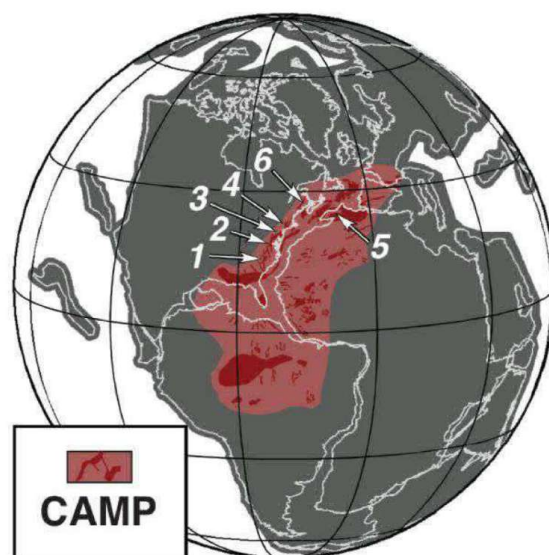


Fig. 1. Location and extent of CAMP magmatism within the Pangean supercontinent. Earliest Jurassic plate configuration showing distribution of the CAMP [based on (36, 40)], including the total areal distribution (pink) and preserved remnants of CAMP (dark red) [based on (5)], and the location of the studied basins: 1, Deep River, North Carolina, USA; 2, Culpeper, Virginia, USA; 3, Gettysburg, Pennsylvania, USA; 4, Newark, New Jersey, USA; 5, Argana, Morocco; and 6, Fundy, Nova Scotia, Canada.

¹Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. ²Lamont-Doherty Earth Observatory of Columbia University, Palisades, NY 10964, USA. ³Department of Earth and Planetary Sciences, Rutgers University, Piscataway, NJ 08554, USA. ⁴Department of Earth and Environmental Sciences, Rutgers University, Newark College of Arts and Sciences, Newark, NJ 07102, USA. ⁵9 Dexters Lane, Grand Manan, New Brunswick E5G 1A1, Canada. ⁶Department of Geosciences, Stony Brook University, Stony Brook, NY 11794, USA. ⁷Département des Sciences de la Terre, Université Mohammed Premier Oujda, 60000 Oujda, Morocco.

*Present address: Department of Terrestrial Magnetism, Carnegie Institution for Science, Washington, DC 20015, USA.

†Corresponding author. E-mail: tblackburn@ciw.edu

dating the Triassic and Early Jurassic stratigraphy (Fig. 2). However, coarse-grained zircon-bearing flows in the CAMP are relatively rare. Therefore, we also have dated sills that are either physically connected to flows as feeders or can be geochemically linked with stratigraphically constrained flows (18, 19). In both North American and Moroccan basins, stratigraphic superposition of basalt flows combined with trace element geochemistry provides a relative time scale for the geochemical evolution of CAMP basalts (11, 20–25) (Fig. 3A). This same geochemical trend is also observed in the dated stratigraphically unconstrained units (Fig. 3A). The shared geochemical evolution in CAMP magmas permits correlation between units that share a common trace element signature, allowing the U-Pb date for a stratigraphically unconstrained intrusion to effectively date the horizon of a geochemically similar and stratigraphically constrained basalt flow. This composite geochronologically dated stratigraphic section is used to test the astrochronological time scale for the Newark basin.

Testing the Astrochronological Time Scale for the Late Triassic

The quasiperiodic variations in Earth's orbit, axial direction, and tilt known as Milankovitch cycles result in corresponding variations in the amount and distribution of sunlight reaching Earth. The resulting forcing on climate and/or ocean circulation can influence the characteristics of sediments deposited within a basin, which may ultimately be preserved within the rock record as cyclical variations in rock lithology, chemistry, or isotopic composition (26). CAMP lava flows within the Newark basin of eastern North America (Fig. 2) are interbedded with strikingly cyclical lacustrine strata that have long been hypothesized to be paced by orbital forcing (27). Astrochronologic models for these sequences have been used to estimate the time represented by the sedimentary rocks between CAMP flows, placing high-precision [± 20 thousand years (ky)] estimates on the total duration of the CAMP at between 580 and 610 ky (9, 28). This proposed "floating" astronomical time scale (ATS) has been met with scrutiny because of the possibility that Earth experienced a different orbital forcing deep in geologic time due to differences in the state of the Earth-Moon system and solar systems dynamics. Further skepticism has focused on whether orbitally forced climatic effects are both accurately recorded and recognized in the geological record. The most straightforward test of the reliability of the ATS is to compare the time durations between basalt flows estimated by orbitally tuning the sedimentary rocks with differences between the zircon U-Pb dates of the flows.

The Orange Mountain Basalt flows are the oldest CAMP lavas in the Newark basin. Although authigenic zircons were not recovered from the Orange Mountain Basalt samples, the flow sequence is partly intruded and fed by an extension of the geochemically identical Palisade sill [201.520 $\pm$ 0.034 million years ago (Ma)] (19).

Based on the ATS for the Newark basin, the overlying flows of the Preakness Basalt should be 250 $\pm$ 20 ky younger than the Orange Mountain Basalt (and Palisade sill) (9). The U-Pb date of the Preakness Basalt (201.274 $\pm$ 0.032 Ma) results in a difference of 246 $\pm$ 47 ky, consistent with the astrochronological estimate. A second interval within the Newark ATS, that again uses the Preakness basalt, can be tested by correlating the Butner diabase from the Deep River Basin (200.916 $\pm$ 0.064 Ma) to the stratigraphically highest and geochemically similar Hook Mountain Basalt (Fig. 3A). The ATS estimate for the time interval be-

tween the base of the Preakness and the base of the Hook mountain basalts is 350 $\pm$ 20 ky, a duration consistent with the difference between U-Pb dates for the Preakness Basalt and the Butner sheet of 358 $\pm$ 72 ky.

A more complete test of the ATS is provided by comparing the astronomical time scale to each of the CAMP flows and intrusions dated here. Using the relative stratigraphy of preserved CAMP flows and the geochemical correlations (Fig. 3A), each stratigraphically unconstrained CAMP intrusive can be correlated to the astronomically tuned Newark Basin. By correlating to the

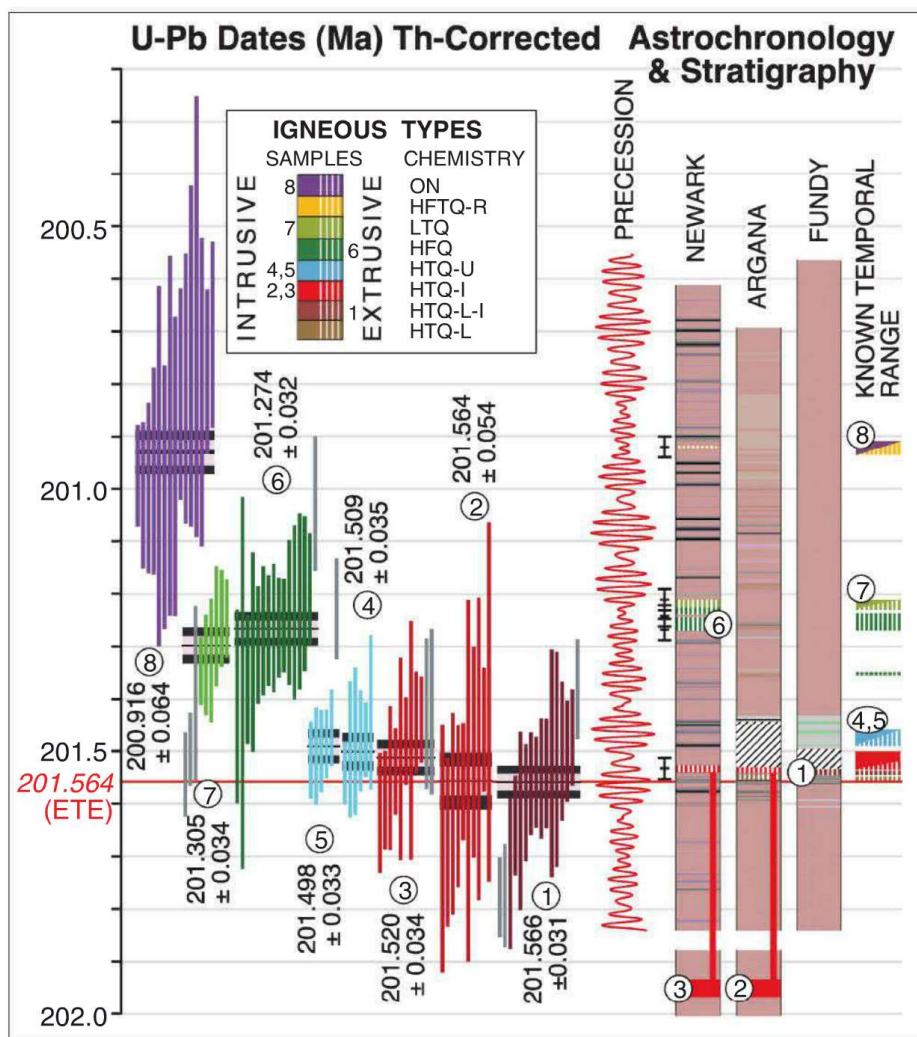


Fig. 2. Zircon U-Pb intercalibration of CAMP volcanism and Early Jurassic Late Triassic astrochronology. Quasiperiodic precessional cyclicity (red) used to generate the ATS (9, 28). Within the stratigraphic sections (right), black- and white-hatched regions mark depositional hiatuses, and error bars next to each basalt mark each unit's timing and uncertainty calculated from the ATS. Colored vertical bars (left) represent the ^{238}U - ^{206}Pb Th-corrected dates (18) for single-crystal zircon analyses at 2 σ , where color indicates basalt chemistry (legend). Legend abbreviations: HTQ, high-titanium quartz normative, includes the lower (L), lower-intermediate (L-I), intermediate (I), and upper (U) units; HFQ, high-iron quartz normative; LTQ, low-titanium quartz normative; HFTQ-R, high-iron-titanium quartz normative or recurrent unit; and ON, olivine normative units. Grayed analyses are excluded from calculated mean dates (18). Sample numbers: 1, North Mountain Basalt; 2, Amelal sill; 3, Palisades sill; 4, York Haven intrusive; 5, Rapidan intrusive; 6, Preakness Basalt; 7, Rossville intrusive; 8, Butner intrusive. Reported dates and black horizontal line mark the weighted mean date, and the outer horizontal box marks the 2 σ uncertainty. Tabulated data, including a second dated North Mountain Basalt sample, are reported in table S1 (18).

Newark section, each horizon dated by U-Pb can be placed within the astrochronologic time scale and assigned an estimated time since the ETE (i.e., the extinction horizon that cannot be directly dated by geochronology) (Fig. 3B). Comparing the U-Pb geochronology and the durations between the ETE and the dated flows implied by the astronomical tuning reveals that the two chronologies agree well given the analytical uncertainties in the U-Pb dates and the ± 20 ky uncertainties assigned to the astrochronology. Combining the two data sets (Fig. 3B), the U-Pb dates anchor the higher-precision astrochronology in absolute time, and a least-squares optimization can be used to simultaneously solve for the absolute date of the ETE as well as those of the dated samples that best fit the relative and absolute timing constraints (table S2) (18). This approach yields an estimate of the ETE of $201.564 \pm 0.015/0.22$ Ma, with a χ^2_{red} (mean square weighted deviation) of 1.3, where the first uncertainty reflects analytical uncertainty only and the second includes uncertainty in the ^{238}U , ^{232}Th , and ^{230}Th decay constants. U-Pb geochronologic data are therefore consistent with our geochemical model and assumed correlations and validates the use of the ATS for high-resolution astrochronology for the CAMP and ETE time interval. This further indicates that orbital signals were faithfully recorded in these lacustrine strata and that solar system dynamics along with the duration of precession cycles 200 Ma can be reliably modeled (29).

The Relationship Between the Eruption of CAMP and the End-Triassic Extinction

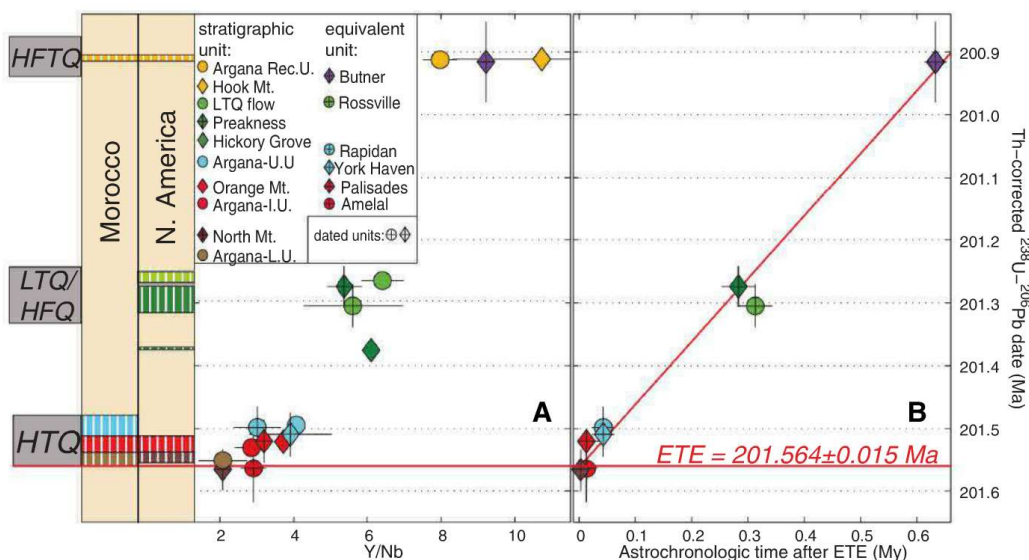
Integration of geochronologic, geochemical (11, 21–23, 25, 30), paleontological (9, 11, 31), and magnetostratigraphic (25, 32, 33) data for the Newark, Fundy, and Argana basins provide the basis for a high-resolution chronology of the earliest known CAMP eruptions and the extinction event (Fig. 4). In each of these sections, the magnetic

polarity chron E23r is observed below the sporomorph turnover that marks the terrestrial ETE. The assumed global synchronicity of both magnetic reversals and mass extinctions at the thousand-year level suggests that a common time duration is shared between the horizons marking the base of the E23r and the ETE. If we assume to first order that the thickness of lacustrine strata between these horizons is a proxy for time, the thickness of sedimentary rocks defines the relative duration between the ETE and the lowermost basalts in each basin (Fig. 4). This relative chronology implies that the base of the North Mountain Basalt of the Fundy basin is older than the base of the Orange Mountain Basalt of the Newark basin, whereas the lowermost basalt within the Argana basin (Tasguint Basalt) is older still than the North Mountain Basalt, placing the Tasguint basalt as the oldest CAMP unit. Time estimates on the sedimentary interval from E23r to the lowermost basalt are provided through integration with the astrochronologic time scale for the Newark basin. Within the Newark basin, the base of E23r to the ETE coincides with one climatic precession cycle (2), thus permitting the precession cycle duration to be correlated to the E23r to ETE interval in both the Fundy and Argana basins (Fig. 4). For a 5-million-year duration of the precession parameter, the average climatic precession cycle is 21.1 ± 6.1 ky (2σ), with a range of 10 to 31 ky. For the Late Triassic–Early Jurassic (200 Ma), that would be $\sim 19.8 \pm 5.7$ ky due to the recession of the Moon (29). Using this model, the ETE is about 13.2 ± 3.8 ky older than the Orange Mountain Basalt (range of 12.9 to 13.5 ky, depending on the section). The base of the North Mountain Basalt of the Fundy basin is about 10.0 ± 2.9 ky older than the base of the Orange Mountain Basalt, which is 3.2 ± 0.9 ky younger than the ETE, and the base of the Tasguint Basalt is 13.2 ± 3.8 ky older than the Orange Mountain and 3.2 ± 0.9 ky older than the North Mountain Basalt.

Both the turnover in sporomorph taxa marking the terrestrial ETE and an interval of post-ETE strata is observed below the lowermost basalts in both the Newark and Fundy basins, implying that within these basins CAMP basalts postdate the extinction. The palynological turnover is, however, not observed within the Argana basin, where Triassic sporomorphs are continually observed within sedimentary rocks up to the base of the Tasguint basalt (11). The combined evidence of (i) the older age of the Tasguint basalt relative to basalts in the Newark and Fundy basins, (ii) the astrochronologic time constraints on sediment deposition for the E23r-basalt interval, and (iii) the presence of Triassic sporomorphs beneath the Tasguint basalt, all place the extinction horizon at the base of or within the Tasguint basalt, consistent with the model by Deenen and others (25) but conflicting with chronology of Marzoli and others (11). This chronologic framework permits a causal relationship between CAMP and the ETE to be made at a high level of precision.

Although this temporal link between the eruption of CAMP and ETE strongly imply a causal relationship, how the eruption of CAMP induced a global extinction remains unclear. An emerging model requires the near-instantaneous eruption of large volumes of magma ($\sim 8 \times 10^5 \text{ km}^3$) in order to explain (i) the apparent increase of atmospheric pCO_2 values (12, 13, 34); (ii) the near collapse of coral reefs and absence of carbonate deposition, both thought to be triggered by the decrease in the pH of ocean seawater (14, 15); and (iii) the approximately 5–8.5 per mil negative excursion in organic $\delta^{13}\text{C}$ values observed in both marine and terrestrial records, suggesting a large input of isotopically light carbon into the atmosphere (35–37). The subsequent biologic recovery from this extinction is marked within marine sections at the Triassic–Jurassic Boundary (TJB) (38), which is defined at its Global Boundary Stratotype

Fig. 3. Geochemical evolution of CAMP magmas and the comparison between astrochronologic and geochronologic time scales. (A) Geochemical evolution for CAMP units is observed in relative time (stratigraphic column, far left) and in geochronologic time. Included for completeness is the undated Hickory Grove Basalt in the Culpeper basin of Virginia. **(B)** Using a geochemical correlation between stratigraphically constrained basalts and the dated intrusives (equivalent units), U-Pb dates from all dated CAMP units are correlated to the Newark basin and assigned an elapsed time since the ETE. Within the U-Pb and astrochronologic uncertainties, a least-squares optimization reaffirms the reliability of the astrochronologic time scale and the assumptions associated with the geochemical correlation.



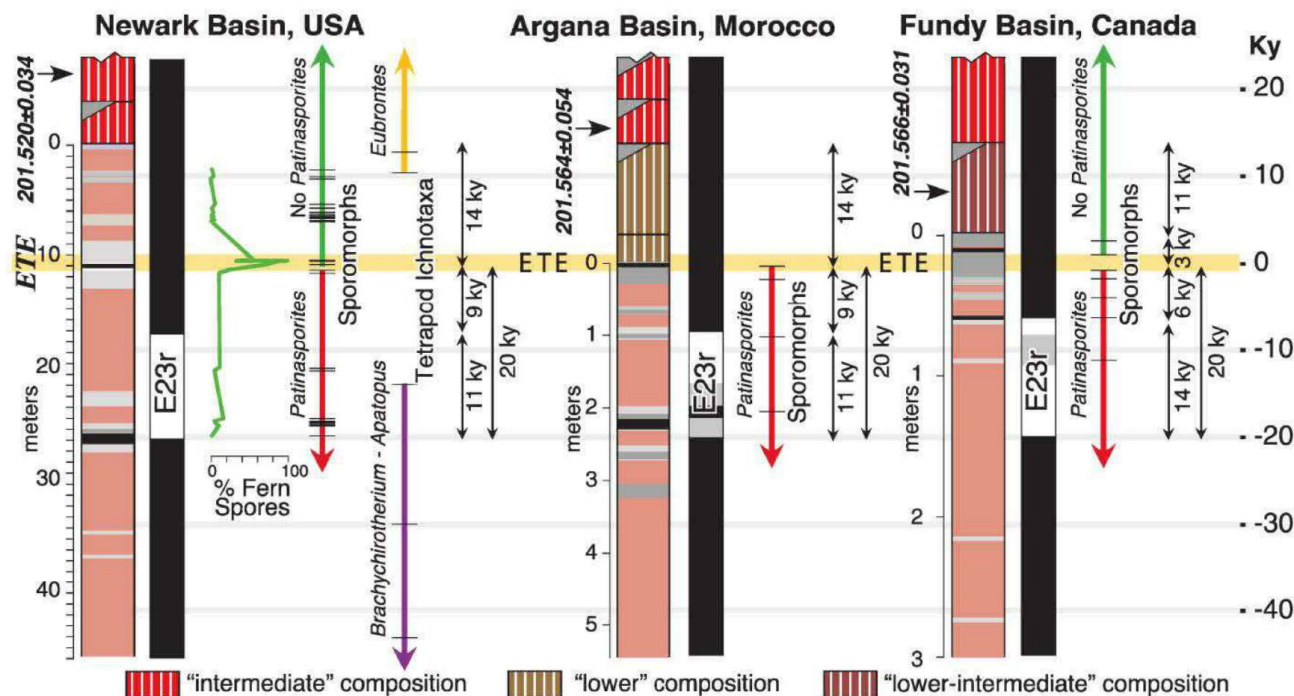


Fig. 4. Integration of geochronologic, magnetostratigraphic, and palynologic data sets for the stratigraphic interval from E23r to the lowermost CAMP basalts in the Newark, Argana, and Fundy basins. Basalt U-Pb dates and magnetic reversal E23r permit correlation between sections. Basalt color correlates to chemistry. Labeled black arrows in-

dicate the astrochronologic time estimates in thousands of years (ky). Palynological data reveal a turnover (green to red arrow transition) in sporomorphs beneath the lower basalt of the Newark and Fundy basin, marking the ETE (yellow horizontal line) at a horizon below the basal CAMP in these basins.

Section and Point (GSSP) as the first appearance of the ammonite *Psiloceras spelae* (39). At the GSSP and throughout northern Europe (35), correlation to the Newark Basin and the ATS (36) places the TJB at $\sim 100 \pm 40$ ky after the ETE (where the large uncertainty derives from problems correlating the marine and terrestrial astrochronologic cycles) and after the eruption of the older, upper high-titanium quartz normative (HTQ-U) basalts. This timeline implies that the biologic recovery associated with the TJB was under way even as subsequent CAMP eruptions and associated pulsed increases in atmospheric CO_2 (13) were occurring ~ 60 ky (HTQ-U), ~ 270 ky [high-iron quartz normative (HFQ-low-titanium quartz normative (LTQ))], and ~ 620 ky [high-iron-titanium quartz normative (HFTQ-R)] after the extinction event (Fig. 3). Because our geochronologic data resolve four discrete CAMP pulses, we propose that it is the first pulse of the "lower" and "intermediate" HTQ chemical groups (Fig. 4) that erupted coincident with and immediately after the ETE that caused the global extinction.

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Supplementary Materials

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 Materials and Methods
 Supplementary Text
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Annually Resolved Ice Core Records of Tropical Climate Variability over the Past ~1800 Years

L. G. Thompson,^{1,2*} E. Mosley-Thompson,^{1,3} M. E. Davis,¹ V. S. Zagorodnov,¹ I. M. Howat,^{1,2} V. N. Mikhalevko,⁴ P.-N. Lin¹

Ice cores from low latitudes can provide a wealth of unique information about past climate in the tropics, but they are difficult to recover and few exist. Here, we report annually resolved ice core records from the Quelccaya ice cap (5670 meters above sea level) in Peru that extend back ~1800 years and provide a high-resolution record of climate variability there. Oxygen isotopic ratios ($\delta^{18}\text{O}$) are linked to sea surface temperatures in the tropical eastern Pacific, whereas concentrations of ammonium and nitrate document the dominant role played by the migration of the Intertropical Convergence Zone in the region of the tropical Andes. Quelccaya continues to retreat and thin. Radiocarbon dates on wetland plants exposed along its retreating margins indicate that it has not been smaller for at least six millennia.

Ice cores from high-altitude tropical glaciers offer long-term perspectives on the variability of precipitation, temperature, aridity, and atmospheric and sea surface conditions at low latitudes. Most meteorological and climatic disturbances affecting Earth's surface and lower atmosphere originate in or are amplified by ocean-atmosphere interactions in tropical latitudes. As Earth's "heat engine," the warmest atmospheric and sea surface temperatures (SSTs) occur there. This energy drives intense convective precipitation and is crucial for the evolution of phenomena such as El Niño–Southern Oscillation (ENSO), the monsoonal systems of Asia and Africa, and, on intraannual time scales, hurricanes and other tropical disturbances that distribute equatorial heat energy poleward. ENSO dominates tropical climate variability. It is linked to the position of the Intertropical Convergence Zone (ITCZ), and its associated teleconnections affect the strength and direction of air masses and storm tracks, variations in convective activity that control flooding and drought, and modulation of tropical storm intensities. There are few high mountain glaciers in these regions

and even fewer that preserve detailed histories of this variability. Unfortunately, most are now rapidly shrinking, and unique records are being lost. The potential impacts on water resources have social and economic consequences that underpin

the imperative to understand the drivers and responses of past and present tropical climate variability.

The first ice cores drilled from the Quelccaya ice cap (QIC) [13°56'S, 70°50'W, 5670 m above sea level (masl)], in 1983 (1–3), could not be returned frozen to the laboratory and instead were cut into samples that were melted and bottled in the field. In 2003, two additional cores were drilled to bedrock on the QIC (Fig. 1). The Summit Dome (5670 masl) core (QSD, 168.68 m) and the North Dome (5600 masl) core (QND, 128.57 m) were returned frozen and are stored at –30°C at Ohio State University's Byrd Polar Research Center. Minimal postdepositional reworking of the snow surface, even during the wet season, results in the distinct annual layers (fig. S1A) used to reconstruct an ~1800-year climate history. Details about the construction of the time scale, extracting annually resolved information, and reconstructing the net annual accumulation are provided in the supplementary text. The oxygen isotopic ratio ($\delta^{18}\text{O}$) records for the 2003 QSD and QND cores, separated by 1.92 km, are highly correlated ($r = 0.898$, $P < 0.0001$ for decadal averages; table S1). In light of their similarity,



Fig. 1. Location of the QIC, Peru, and other ice fields and features discussed in the text. Also included are the upper (500 hPa) and lower (850 hPa) level atmospheric circulation in the austral summer [December–January–February (DJF)] (38).

¹Byrd Polar Research Center, The Ohio State University, Columbus, OH 43210, USA. ²School of Earth Sciences, The Ohio State University, Columbus, OH 43210, USA. ³Department of Geography, The Ohio State University, Columbus, OH 43210, USA. ⁴Institute of Geography, Russian Academy of Sciences, Moscow, Russia.

*Corresponding author. E-mail: thompson.3@osu.edu

documented in the supplementary text, all subsequent discussions are based on the QSD core.

These records (Fig. 2 and fig. S2), based on freshly cut ice samples, are precisely dated and include $\delta^{18}\text{O}$ and concentrations of insoluble dust and major anions (F^- , Cl^- , SO_4^{2-} , and NO_3^-) and cations (Na^+ , NH_4^+ , K^+ , Mg^{2+} , and Ca^{2+}). The reproducibility of decadal averages of $\delta^{18}\text{O}$ (1983 Core 1 versus the 2003 QSD core) from 1000 to 1982 CE (fig. S3A) is excellent ($r = 0.856$, $P < 0.0001$). Details of the reproducibility of $\delta^{18}\text{O}$ and net accumulation among the four cores, two in 1983 and two in 2003, over the past 1000 years are in the supplementary text (fig. S3, A and B, and table S1).

The Record

Figure 2 presents the decadal averages of $\delta^{18}\text{O}$, net accumulation, insoluble dust, ammonium (NH_4^+), and nitrate (NO_3^-) measured in the QSD core. Decadal averages of all other species are shown in fig. S2. Because annual resolution is preserved to ~160 m (683 CE) (supplementary materials), the annual net accumulation (A_n) record terminates there. These histories detail changes in climatic and environmental conditions in the tropical Andes over the past 18 centuries.

Four distinct climatic stages are evident. Table S2 presents the average values of all QSD core constituents for the different time periods discussed in the paper. Before 1100 CE, most constituents show little variability, although accumulation and insoluble dust concentrations are slightly higher and decline slowly toward 1100 CE. Simultaneously, $\delta^{18}\text{O}$ becomes modestly depleted in ^{18}O .

The Medieval Climate Anomaly (MCA) is characterized by below-average A_n , consistent with hydroclimate reconstructions for this region (4), and $\delta^{18}\text{O}$ is more variable and modestly enriched [0.26 per mil (‰)] relative to the long-term average (−17.92‰) (table S2). MCA ice contains no visible evidence of surface melting or smoothing of the $\delta^{18}\text{O}$ annual signal that, as discussed below, characterizes the QIC core since 1991. For just 4 decades near the end of the MCA, the QSD core contains high levels of ammonium (NH_4^+) and nitrate (NO_3^-) that are not contemporaneous with $\delta^{18}\text{O}$ evidence of strong warming or any notable increase in A_n . During the MCA, the climate across South America appears highly variable, possibly reflecting the observed dipole in humidity over the northern and southern Amazon Basin (5). The result is that wetter conditions in the northeast tend to be contemporaneous with drier conditions in southern Amazonia. For example, a high-resolution record of El Niño flooding from a marine core off the coast of Peru at 12°S indicates intense aridity from 800 to 1250 CE (6), whereas the Cariaco Basin (Venezuela) titanium record (7) reflects wetter-than-average conditions in the northeast Amazon Basin from 950 to 1450 CE.

The most prominent feature in the QIC record is the Little Ice Age (LIA). Early in the 16th century, concentrations of ammonium, nitrate, calcium, magnesium, and sulfate began to increase

(Fig. 2, fig. S2, and table S2). The period from ~1520 to 1880, identified as the LIA, is characterized by lower $\delta^{18}\text{O}$ values that remained low until the late 19th century. The net accumulation trend is distinctive. In ~1520 CE, A_n increased rapidly but declined abruptly in ~1680 CE from record highs in the Early LIA (1520 to 1680 CE) to record lows (~1800 CE) in the Late LIA (1681 to 1880 CE). Over much of the LIA, concentrations of most ionic species were persistently high but declined rapidly near the end as A_n began to increase.

With the onset of the Current Warm Period (CWP, 1880 CE to the present), both $\delta^{18}\text{O}$ and A_n increased, whereas all aerosol concentrations have remained low over much of the CWP. Over the past 30 years, most aerosol concentrations have increased severalfold primarily because of postdepositional surface melting and percolation through the firn pack. The firn-ice transition is at 17.7 m and corresponds to 1992; however, isotopic smoothing in the top of the record indicates that, since the onset of the melting and retreat of the ice cap in ~1991, meltwater has percolated down to the 1980 level [see figure 4 in (8)].

Linkages with the Tropical Pacific

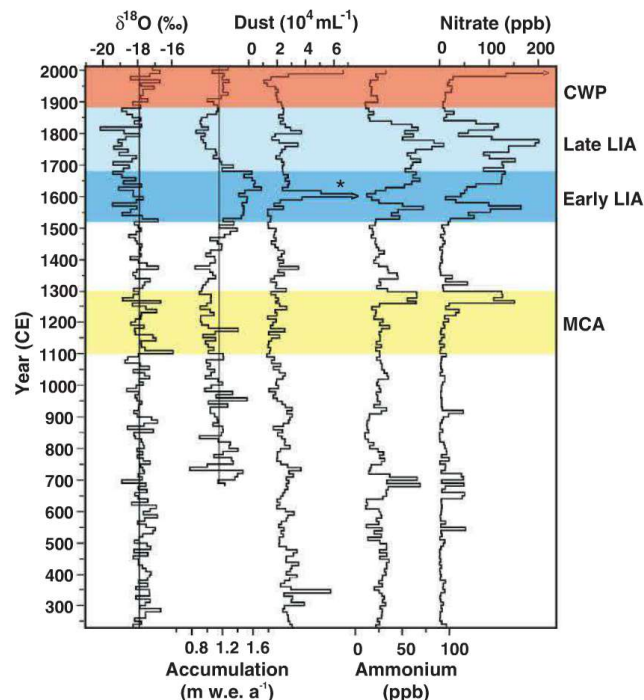
The seasonal temperature range in the tropics is only a few degrees, whereas the seasonal differences in the $\delta^{18}\text{O}$ of Andean snowfall are much larger, often up to 20‰. Because 70 to 80% of the precipitation falling on Quelccaya arrives during the wet season, the $\delta^{18}\text{O}$ history reflects primarily conditions during austral summer. Decadal averages of precipitation amount and $\delta^{18}\text{O}$ are not strongly related (fig. S3). Twenty-one-year running correlations between $\delta^{18}\text{O}$ and A_n alternate between positive and negative values over multiple decades (fig. S4), and very few co-

efficients are significant at the 95% level. Essentially, there is no consistent, long-term statistically significant relationship between the amount of precipitation and its $\delta^{18}\text{O}$ signature. In general, low-latitude isotopic ratios yield a climate signal reflecting a variety of hydrologic and thermal influences in the broad geographic region that supplies moisture to the high glaciated mountains (9).

Although the moisture source for Quelccaya precipitation is primarily from the tropical Atlantic via the Amazon Basin, Vuille *et al.* (10) demonstrated that Pacific SSTs exert the dominant control on interannual $\delta^{18}\text{O}$ variability preserved in tropical Andean ice cores. This occurs via the expansion of the tropical troposphere associated with a warm tropical Pacific and enhanced westerly flow over the tropical Andes or via the shrinkage of the tropical troposphere associated with a cool tropical Pacific, and enhanced easterly flow over the subtropical Andes. These upper-level wind anomalies force the low-level moisture flow over the Andes and thereby link oceanic forcing and climate variability on interannual time scales.

Bradley *et al.* (11) demonstrated that $\delta^{18}\text{O}$ in the Sajama (Bolivia) ice core is more closely linked to SSTs, and hence to ENSO variability, across the equatorial Pacific Ocean even though the moisture source for precipitation is the Atlantic. A $\delta^{18}\text{O}$ composite of three Peruvian ice cores and one Himalayan ice core from 1856 to 1996 CE [see figure 3 in (8)] is strongly linked ($r = 0.73$; $P < 0.0001$, for 5-year moving averages) to the NINO4 extended reconstructed SSTs [ERSST (12)]. This strong relationship between ice core isotopic records throughout the tropics and tropical SSTs likely reflects the dominance of tropical evaporation in determining water vapor flux into the atmosphere (13). This also provides a

Fig. 2. Decadal averages of $\delta^{18}\text{O}$, net accumulation, insoluble dust, ammonium, and nitrate in the QSD ice core. Specific climatological periods discussed in the text are shaded and identified. The asterisk on the dust profile indicates the 1600 CE eruption of Huaynaputina (Peru). ppb indicates parts per billion; m w.e. a^{-1} indicates meters of water equivalent per year.



likely explanation of the large-scale isotopic links among low-latitude, high-altitude ice core records (14).

Figure 3A illustrates the spatial distribution of the correlations between the annual QSD $\delta^{18}\text{O}$ and ERSST records between 80°N and 80°S from 1870 to 2009 CE (15, 16). The 2003 ice core record was extended to 2009 by using subsequently collected pit and shallow core samples. The annual $\delta^{18}\text{O}$ values of QIC precipitation are positively related to equatorial SSTs in the mid to eastern tropical Pacific Basin (solid line encloses $P < 0.001$). Correlations between the thermal-year (July to June) averages of $\delta^{18}\text{O}$ in the QSD core and ERSSTs in the NINO4, NINO3.4, and

NINO3 regions were examined. The NINO4 SSTs are most strongly related to the QSD $\delta^{18}\text{O}$ ($r = 0.55$ for annual data; $r = 0.61$ for 3-year averages; $P < 0.0001$).

Figure 3B shows the 3-year running means of the NINO4 SST index (12) and QSD $\delta^{18}\text{O}$ for thermal years 1870–1871 to 2009–2010. Given their strong correlation ($r = 0.61$, $P < 0.0001$; fig. S5), the resulting SST- $\delta^{18}\text{O}$ transfer function ($\text{SST}_{\text{NINO4}} = 0.19 \times \delta^{18}\text{O} + 29.64$) is used to reconstruct a contemporaneous SST history for the NINO4 region (Fig. 3C) for the past 18 centuries. The reconstructed SSTs range between 25.8° and 26.6°C (decadal averages) or 25.0° to 27.1°C (annual data) with temperatures in the region depressed by

0.2°C during the LIA (1520 to 1880 CE) relative to the 20th-century average.

This reconstruction assumes that the QSD $\delta^{18}\text{O}$ -NINO4 SST relationship has been stationary over the past 1800 years, which is not the nature of climate processes. The spatial distribution of the most highly correlated fields is not stationary through time (fig. S6). From near the end of the LIA (1870 CE) to 1900 CE, the enriched values of QSD $\delta^{18}\text{O}$ are strongly related to higher SSTs in the eastern Pacific region along the equator and extending southward. Note the strong positive correlation in the equatorial Atlantic at this time as well. Over the next 50 years (1901 to 1950 CE), the $\delta^{18}\text{O}$ -SST correlations weaken, and the region of strongest positive correlations moves northward. In the past ~60 years (1951 to 2009 CE) during which anthropogenic forcing has increased (17), the region of strongest correlation increases in coherence and migrates north of the equator. The movement of the fields of significant $\delta^{18}\text{O}$ -SST correlation into the Northern Hemisphere over the CWP likely reflects the post-LIA northward migration of the ITCZ over the tropical Pacific Basin, consistent with other evidence, including lake sediments from central equatorial Pacific islands (18).

A comprehensive review of various proxy indicators from diverse tropical sites (19) suggests a widespread southward migration of the ITCZ over the Holocene (their figure 12-5) affecting the Pacific, Indian, and Atlantic basins. In general, wetter conditions in the northern tropics gave way to more arid conditions in the late Holocene, whereas the southern tropics experienced the reverse trend. This plus evidence from coral records from the tropical Pacific (20) indicate that the movement of the ITCZ was coherent between the Atlantic and Pacific oceans (19). The climatological controls on snow accumulation and the isotopic chemistry of precipitation on the QIC are likely to change with the position of the ITCZ. Thus, the QSD records should provide additional details of the ITCZ migration.

ITCZ Migration and Tropical Linkages

As discussed above, the tropical eastern Pacific SSTs and atmospheric circulation influence the stable isotopic composition of the precipitation falling on Quelccaya, as well as on other central Andean glaciers (11, 21). However, much of the precipitation falling on the eastern side of the Peruvian Andes is produced by deep convection in the tropical Amazon Basin during the austral summer. Much of the water vapor originates in the tropical Atlantic, as it has since the Last Glacial Maximum. This is confirmed by mountain snow lines that were tilted toward the Amazon Basin then as they are now (22).

Elevated concentrations of nitrate (NO_3^-) and ammonium (NH_4^+) and ^{18}O depleted isotopes in the QSD during the LIA are contemporaneous with decreasing percentages of Ti in the Cariaco Basin record off the coast of Venezuela at 10°N (Fig. 4). Reduced Ti concentrations indicate

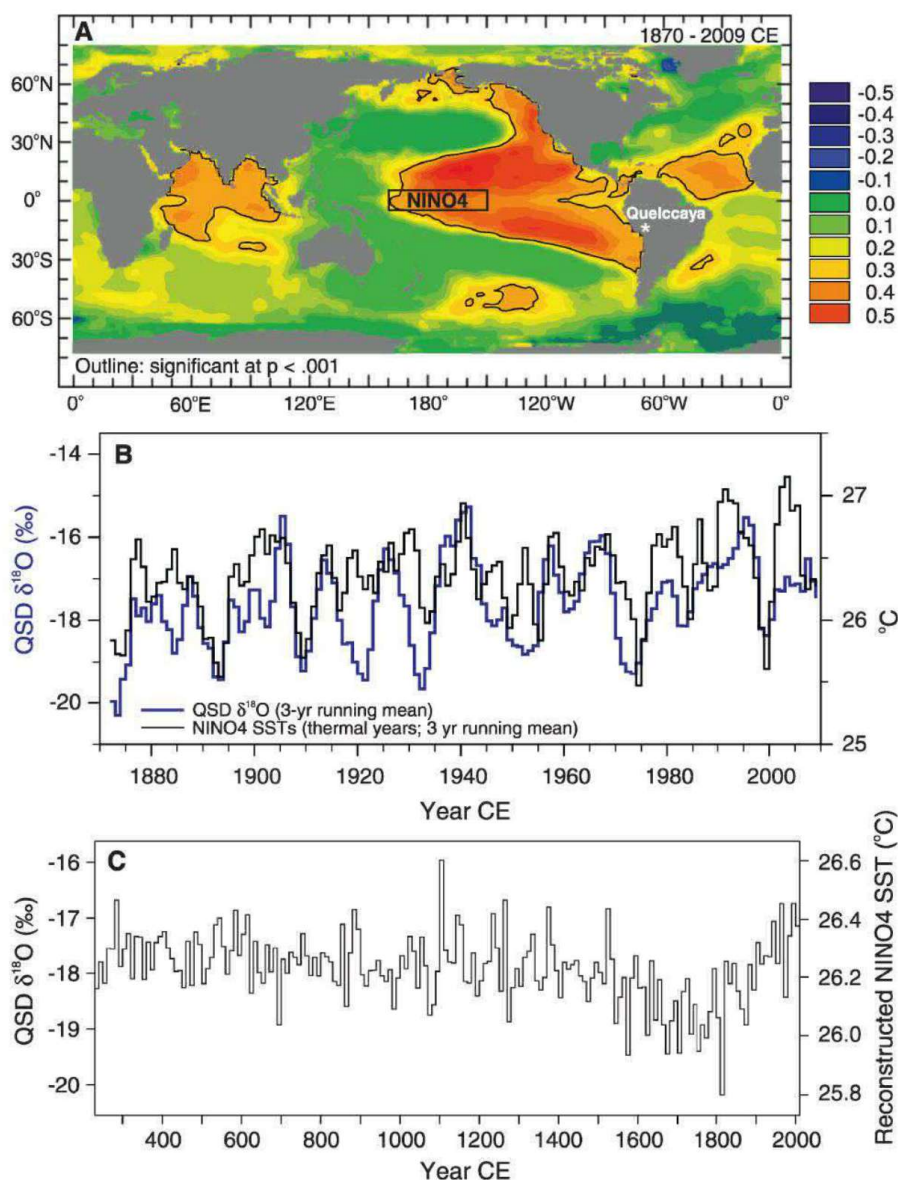


Fig. 3. QSD $\delta^{18}\text{O}$ and SST comparisons. (A) Spatial distribution of correlations between the annual QSD $\delta^{18}\text{O}$ and ERSST records [(15, 16) and <http://iridl.ldeo.columbia.edu/SOURCES/.NOAA.NCDC.ERSST/.version3b/.sst/>] between 30°N and 30°S from 1870 to 2009 CE. (B) Three-year running means of the thermal-year averages of QSD $\delta^{18}\text{O}$ and SSTs in the NINO4 region [(12) and www.esrl.noaa.gov/psd/gcos_wgsp/Timeseries/Nino4] from 1871 to 2009 CE. (C) ~1800 year SST history reconstructed by using the QSD $\delta^{18}\text{O}$ /SST regression equation (fig. S5) for data in (B).

decreased precipitation and runoff from the northeast coast of tropical South America (7). Conversely, elevated NO_3^- and NH_4^+ likely reflect more moist conditions to the south over the Amazon Basin. These aerosol species originate in part as products from ammonia (NH_3), a gas produced by biological activity in the soil and vegetation in the Amazon Basin (23, 24). NO_3^- and NH_4^+ are correlated over the entire QSD core ($r = 0.76$, $P < 0.0001$ for decadal averages, from 230 to 1990 CE) and covary most strongly ($r = 0.92$, $P < 0.0001$) over the LIA. QSD NH_4^+ and therefore NO_3^- concentrations are significantly correlated with precipitable water in the southwest Amazon Basin from 1949 to 2002 (Fig. 5).

Both the Cariaco Basin and the Caribbean Sea lie along the path of moisture flow to the central Andes and experienced lower SSTs during the LIA (25, 26). The Quelccaya $\delta^{18}\text{O}$ values also suggest lower SSTs in the tropical Pacific (Fig. 3C) at this time, indicating a LIA cooling in both ocean basins. Hydrological conditions were more regionally variable, and the QSD records suggest more complex large-scale linkages, possibly reflecting the recent northward migration of the ITCZ. However, over the Amazon Basin the ITCZ is not well defined but appears as a wide area of continental convection that is connected with the convergence zones over both the tropical Pacific and Atlantic oceans. Thus, its migration is not a simple shift into subtropical latitudes (27), and the associated precipitation anomalies are more complex. For example, lake (28) and speleothem (29) records in northern Peru indicate wet conditions, whereas speleothem records in northeastern Brazil (30) suggest dry conditions during the LIA.

Water levels measured in Lake Titicaca show reasonable similarities with the Quelccaya composite A_n before 1975 (fig. S7). Thereafter, mass wasting on the QIC has led to decreasing net accumulation. Marengo (5) demonstrated that even under current warmer conditions precipitation cycles in the northern and southern Brazilian Amazon Basin are out of phase, with both showing precipitation trend reversals in the mid-1940s and 1970s. Both QSD A_n and Lake Titicaca water level records closely reflect the timing and magnitude of changes in the Northern Amazonia Rainfall Index from 1929 to 1998 [figure 3 in (5)], which the author links to changes in oceanic and atmospheric circulation patterns and SSTs in the tropical central and eastern Pacific. These patterns argue not only that the QSD A_n represents the larger-scale regional pattern in Amazonian precipitation but that the NO_3^- and NH_4^+ signals likely originate in the Amazon Basin northeast of the QIC (Fig. 5) as a result of northeast airflow at the 850-mbar level (Fig. 1). Figure 4 illustrates that over the past ~1800 years the LIA is the most prominent feature in three ice-core records (Huascarán, Quelccaya, and Illimani; Fig. 1) collected along a north-to-south Andean transect. The relationships among these records suggest large-scale regional climate variability that requires multiple ice cores

to disentangle the complex atmospheric and hydrological dynamics of the region.

The QIC accumulation history represents a much larger regional signal of precipitation variability. A_n on Quelccaya (Fig. 2) was well above average in the first half of the LIA (1520 to 1680 CE) and much reduced during the second half (1680 to 1880 CE). The A_n record is very similar to the 400-year climate history based on pollen (Poaceae/Asteraceae or P/A ratios) from the Sajama ice cap

(Bolivia) 350 km to the southwest (31) and is consistent with nearby records of LIA glacier advance (32). The highest A_n rate in the QSD record is contemporaneous with the maximum LIA glacial extent (1630 to 1680 CE) in Bolivia and Peru, suggesting a cool and humid period followed by drier and gradually warming conditions as glaciers retreated. The humidity of the Bolivian/Peruvian Altiplano is reflected over long time scales by the nearby Lake Titicaca record (33).

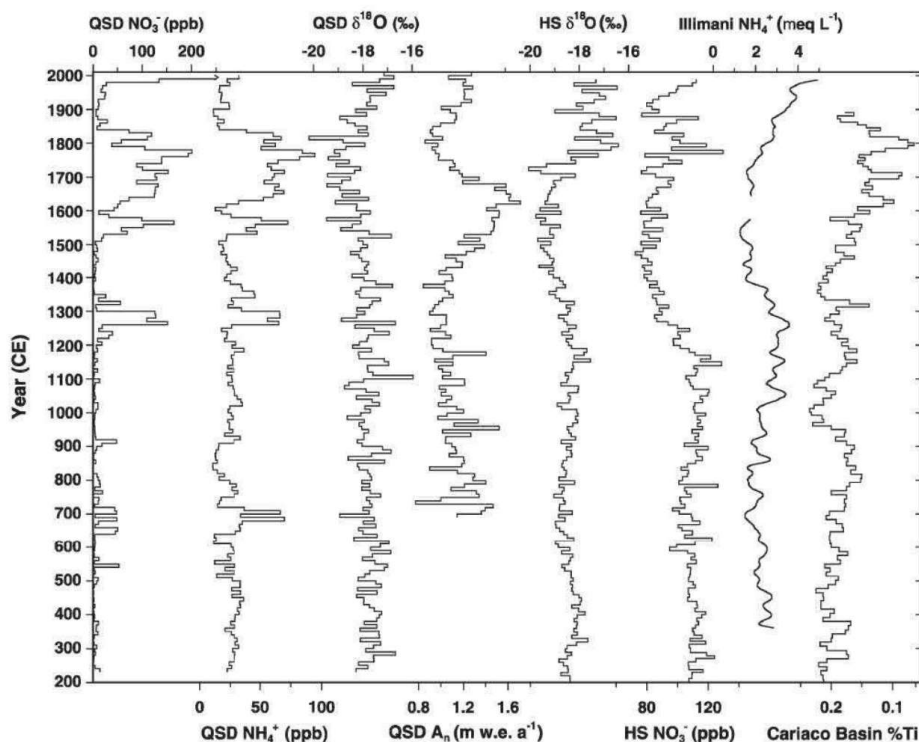


Fig. 4. Decadal averages of nitrate, ammonium, $\delta^{18}\text{O}$, net accumulation from QIC's QSD, $\delta^{18}\text{O}$ and nitrate from Huascarán in northern Peru (34), and Ti from Cariaco Basin (7). Note reversed axis for Ti. Illimani ice cap ammonium data are courtesy of M. Schwikowski (Paul Scherrer Institut).

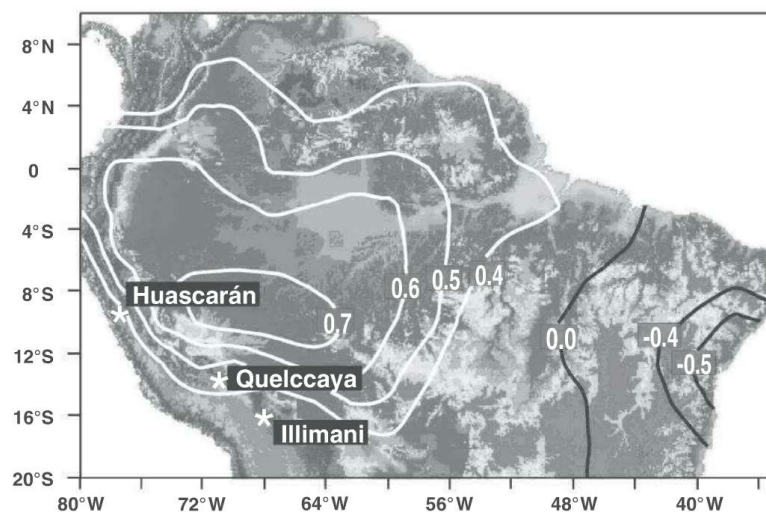


Fig. 5. Correlation fields between National Centers for Environmental Prediction/National Center for Atmospheric Research summertime (December to February) precipitable water (39) and annual ammonium concentrations from 1949 to 2002 in the QSD.

Thus, the QSD A_n record reflects larger regional changes in precipitation and indicates that Lake Titicaca's water level was likely above average from ~1520 to ~1680, after which it declined to much lower levels until rising again in the 20th century.

Throughout the LIA, elevated concentrations of NO_3^- and NH_4^+ on Quelccaya are contemporaneous with reduced Ti deposition in the Cariaco Basin (7) (Fig. 4), which indicates arid conditions in the northeast portion of the Amazon Basin. If the relationship between precipitable water and QSD NH_4^+ and NO_3^- (Fig. 5) persisted over the LIA, then wetter conditions in the southwest Amazon Basin just upwind of Quelccaya would be contemporaneous with drier conditions in northeast Brazil (30), consistent with a southward migration of the ITCZ (fig. S6), resulting in a region of intense continental convection over tropical Amazon Basin. Strong similarities exist between the oxygen isotope records from Quelccaya and Huascarán to the north; however, the differences between the QSD NH_4^+ record and the Huascarán NO_3^- (34) and Illimani NH_4^+ (24) records tell a different story (Fig. 5), suggesting different sources for these species.

The QIC NO_3^- and NH_4^+ concentrations reflect hydrological conditions in the Amazon Basin northeast of Quelccaya (Fig. 5) with moisture advected to the site by northeasterly winds at the 850 hPa level (Fig. 1) (below the boundary layer). However, the moisture bringing the $\delta^{18}\text{O}$, the NO_3^- deposited on Huascarán (6050 masl), and the NH_4^+ deposited on Illimani (6300 masl) arrives via winds at the 500-hPa level originating from the east-southeast. Because both Illimani and Huascarán are located outside the area where QSD and NH_4^+ are correlated with precipitable

water (Fig. 5), this points to the complexity of precipitation and chemistry patterns in the Andes. Additional linkages are discussed in the supplementary text, but, whatever the ultimate driver, NH_4^+ cannot be interpreted as a large-scale regional temperature recorder in all tropical ice cores.

Modern Retreat of the Margin of the Quelccaya Ice Cap

Nearly annual field observations confirm that since 1978 the QIC has been retreating along its margins (14). Radiocarbon-dated wetland plants exposed by the retreating ice provide temporal constraints on both its advance and retreat. Twenty wetland plants exposed by the retreating margin of the QIC were collected between 2004 and 2007 next to a meltwater lake (site A in Fig. 6) that started forming in 1985 on the west side of the ice cap. Radiocarbon dates for the plants [4676 ± 41 years before the present (yr B.P.)] indicate that the ice cap is smaller than it has been in almost five millennia (14, 35). In 2011 as the ice cap continued to retreat, fresh plant remains were uncovered on the eastern side of the expanding North Lake (site B in Fig. 6), while most of the plants exposed in 2002 had already decayed because of their lack of woody tissue. Radiocarbon dates on these newly exposed plants, still in growth position, average 6298 ± 35 yr B.P. (table S3), ~1600 years older than the plants collected on the west side of the lake, indicating that Quelccaya is now smaller than it was 6000 years ago. Moreover, the plant ages confirm that the advance of the QIC ~6000 years ago was much slower, ~300 m over ~1600 years, than its current rate of retreat, ~300 m in 25 years. $\delta^{18}\text{O}$ is not correlated with net accumulation on decadal and longer time scales (Fig.

2 and figs. S3 and S4) but is highly correlated with SSTs in the eastern equatorial Pacific (Fig. 3, A and B). For the past century, Quelccaya's net accumulation has been above average, while $\delta^{18}\text{O}$ has been enriched (Fig. 2), suggesting that the current retreat is driven in part by its warming environment. The rapidity of Quelccaya's retreat may reflect snow-ice feedbacks that are considered instrumental (36) for rapidly increasing temperature trends near the 0°C isotherm during the 20th century. The accelerating retreat of Quelccaya and other tropical ice fields (8) is consistent with model predictions for vertical amplification of temperature in the tropics (37) and has serious implications for those living in these areas.

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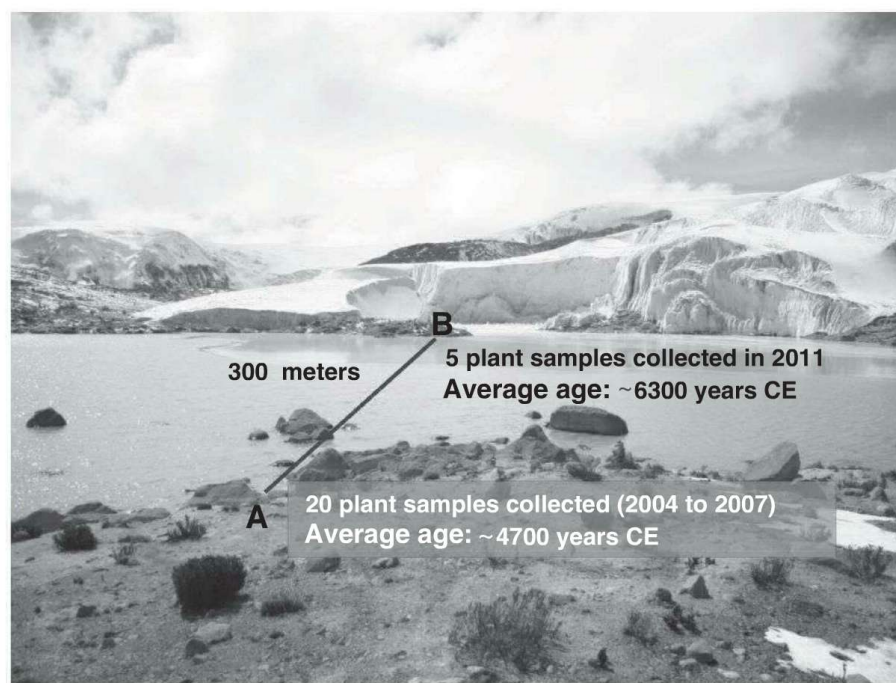


Fig. 6. Locations of recently exposed plants collected along the western side of the QIC.

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Data Center-A for Paleoclimatology: <ftp://ftp.ncdc.noaa.gov/pub/data/paleo/icecore/trop/quelccaya/quelccaya2013.txt>.

Supplementary Materials

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Materials and Methods

Figs. S1 to S6

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REPORTS

An Accurate Geometric Distance to the Compact Binary SS Cygni Vindicates Accretion Disc Theory

J. C. A. Miller-Jones,^{1*} G. R. Sivakoff,^{2,3} C. Knigge,⁴ E. G. Körding,⁵ M. Templeton,⁶ E. O. Waagen⁶

Dwarf novae are white dwarfs accreting matter from a nearby red dwarf companion. Their regular outbursts are explained by a thermal-viscous instability in the accretion disc, described by the disc instability model that has since been successfully extended to other accreting systems. However, the prototypical dwarf nova, SS Cygni, presents a major challenge to our understanding of accretion disc theory. At the distance of 159 ± 12 parsecs measured by the Hubble Space Telescope, it is too luminous to be undergoing the observed regular outbursts. Using very long baseline interferometric radio observations, we report an accurate, model-independent distance to SS Cygni that places the source substantially closer at 114 ± 2 parsecs. This reconciles the source behavior with our understanding of accretion disc theory in accreting compact objects.

The accretion of matter onto a central compact object via a disc powers systems as diverse as active galactic nuclei (AGN), x-ray binaries (XRBs), cataclysmic variables (CVs), and young stellar objects. This universal process directly impacts the surrounding environment by driving powerful outflows and jets. CVs, which comprise a white dwarf accreting via Roche lobe overflow from a low-mass stellar companion, provide excellent laboratories for studying disc accretion. There is a large population of nearby CVs, and during outburst the accretion disc both dominates the observed emission at all wavelengths and exhibits structural changes on accessible time scales.

When the mass transfer rate through an accretion disc is sufficiently high, the disc will remain in a fully ionized, stable state. At lower accretion rates, the resulting lower temperatures allow the recombination of hydrogen, leading to large changes in the disc opacity. This alters the disc cooling mechanism and causes the disc to become thermally unstable, leading to the semi-regular outburst events seen in dwarf nova CVs. The disc instability model (DIM) describes the resulting behavior of the disc as it undergoes a limit cycle, making repeated transitions between a cool quiescent state and a hot, viscous outburst state. With various modifications, the DIM has been successful in both explaining the outbursts

of dwarf novae and providing a basic framework for understanding the outbursts of their more massive analogs, the XRBs (1).

SS Cygni is the brightest and best-studied dwarf nova, with an optical light curve containing close to 500,000 observations stretching back to its discovery in 1896. It shows repeated outbursts with a mean recurrence time of 49 ± 15 days (2). For a distance of ~ 100 pc, the outburst properties can be well reproduced by the DIM when including the effects of a truncated inner disc and small variations in the mass transfer rate from the secondary (3). However, the optical parallax measured by the Hubble Space Telescope (HST) (4–6) places the source at a distance of 159 ± 12 pc, implying that it has the highest absolute visual magnitude of any dwarf nova (7). The mass accretion rate required during outburst to generate this optical luminosity is then too high to be compatible with the DIM, because the mean mass transfer rate becomes high enough that the disc should be persistently in the hot, ionized, stable state. SS Cygni should then appear as a nova-like CV with no outbursts. At a distance of 159 pc, the difference in behavior between SS Cygni and the more stable nova-like CVs with similar binary parameters cannot then be ascribed to a difference in the mean mass transfer rate (8). This would contradict not only the key prediction of the DIM, but our entire understanding of accretion discs in CVs (9) and other accreting systems (10, 11). The only possible explanation would be an enhancement in mass transfer rate during outburst (12), but the required enhancement factor of 150 is implausibly high (13).

Because SS Cygni is known to emit radio emission during outbursts (14), we used very long

¹International Centre for Radio Astronomy Research, Curtin University, G.P.O. Box U1987, Perth, WA 6845, Australia.

²Department of Physics, University of Alberta, CCI5 4-183 Edmonton, AB T6G 2E1, Canada. ³Department of Astronomy, University of Virginia, P.O. Box 400325, Charlottesville, VA 22904, USA. ⁴School of Physics and Astronomy, University of Southampton, Highfield, Southampton SO17 1BJ, UK. ⁵Department of Astrophysics/IMAPP, Radboud University Nijmegen, P.O. Box 9010, 6500 GL Nijmegen, Netherlands. ⁶American Association of Variable Star Observers, 49 Bay State Road, Cambridge, MA 02138, USA.

*Corresponding author. E-mail: james.miller-jones@curtin.edu.au

Table 1. Fitted astrometric parameters for SS Cygni. Uncertainties are 1σ .

Parameter	Fitted value
Right ascension α_0 (J2000)	21:42:42.923121 $\pm$ 0.000006
Declination δ_0 (J2000)	43:35:10.25301 $\pm$ 0.00007
Proper motion in right ascension ($\mu_\alpha \cos \delta$; mas year ⁻¹)	112.42 $\pm$ 0.07
Proper motion in declination (μ_δ ; mas year ⁻¹)	33.38 $\pm$ 0.07
Parallax (mas)	8.80 $\pm$ 0.12
Distance (pc)	114 $\pm$ 2
Reference epoch (MJD)	55,769.0

baseline interferometry (VLBI) to measure a model-independent distance based on trigonometry alone. We observed the source with the Very Long Base-

line Array (VLBA) and the European VLBI Network (EVN), for a total of 10 epochs between April 2010 and October 2012 (15). Each observation

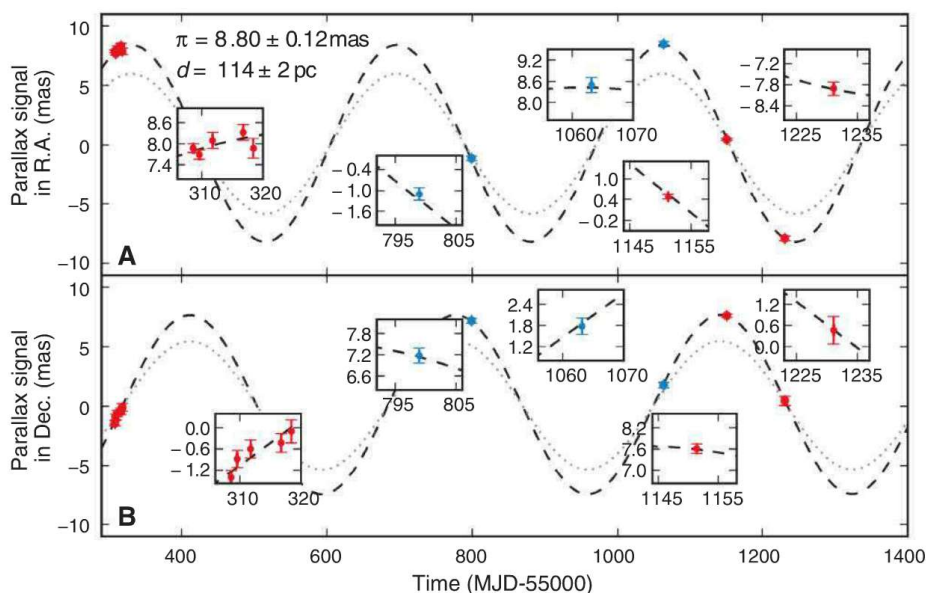


Fig. 1. Parallax signature of SS Cygni. (A) Right ascension; (B) declination. The measured position of the source is plotted against time after subtracting the best-fitting reference position and proper motion given in Table 1. Red points show VLBA measurements; blue points [at modified Julian date (MJD) 55799 and 56063] show EVN measurements. The dashed line shows the best-fitting parallax signal, and the gray dotted line shows the calculated signal for a distance of 159 pc. Insets show zoom-ins of the regions around each point.

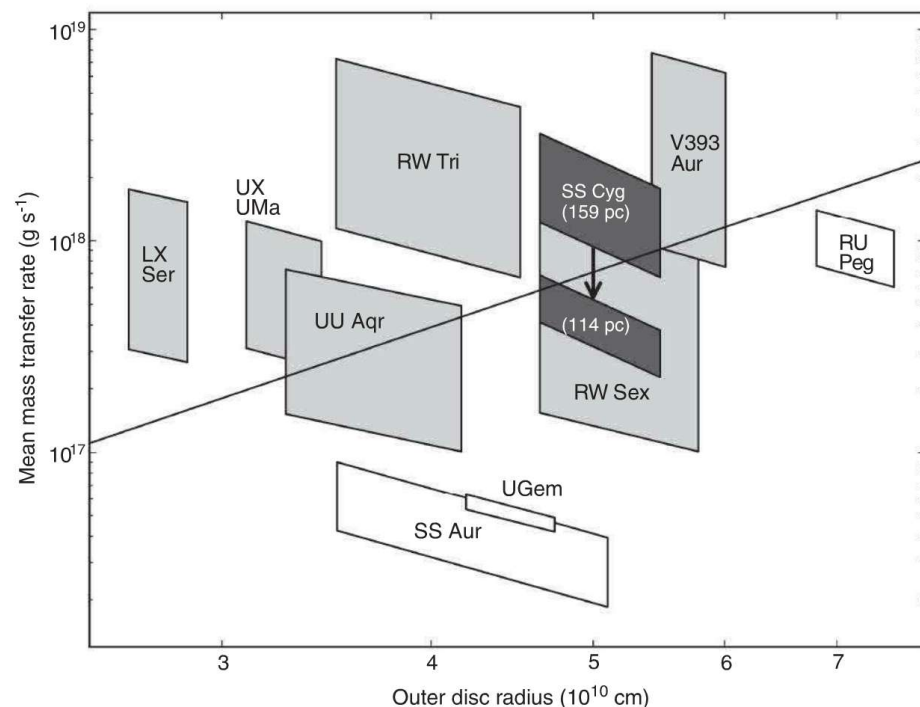


Fig. 2. Calculated mean mass transfer rates as a function of outer disc radius. The critical mass transfer rate above which systems are stable against dwarf nova outbursts is shown as a black line. Boxes show relatively conservative error regions derived from uncertainties on the system parameters. Values for SS Cygni were calculated from the power-law approximation of (7), using the system parameters tabulated in (8). Values for all other systems were taken directly from (8). Dwarf novae are shown in white, nova-like CVs in light gray, and SS Cygni in dark gray, with the arrow indicating the effect of our distance revision.

was triggered by the optical detection of an outburst by members of the American Association of Variable Star Observers (AAVSO). Fitting the measured positions for a reference position, proper motion, and parallax (Table 1), we find a best-fitting distance to SS Cygni of 114 ± 2 pc (Fig. 1), with a reduced χ^2 value of 1.1. The HST distance of 159 pc is ruled out at a very high level of significance (a reduced χ^2 value of 59).

The origin of this discrepancy is unclear. One key advantage of radio parallaxes lies in their use of extragalactic calibrator sources, allowing a direct measurement of the target parallax. By contrast, optical measurements are made relative to nearby stars, so that the mean parallax of the reference stars has to be added to the measured parallax of the target. Therefore, the discrepancy could in principle be due to a problem with one or more of the optical reference stars. However, the difference between our radio parallax and the uncorrected HST optical parallax is more than twice the mean optical reference parallax of 2.23 ± 0.22 mas (milli-arc sec) and is larger than the parallax of every single optical reference star (4, 5). Thus, to reconcile the radio and optical measurements, there would have to be a very large error in one or more of the reference star parallaxes.

Another possible resolution relates to an inherent bias affecting parallax measurements (16). Measurement errors imply that sources at both larger and smaller intrinsic distances could be measured to have a given parallax. However, for a uniform density of sources, the increased volume of a larger spherical shell makes it more probable that for a given parallax measurement the real source distance is larger, and so measured parallaxes tend to be biased high. Although the HST measurement was corrected for the expected bias of 1.6% (5), the uncertainty on such corrections can be quite large (17). The precision of our radio parallax makes it largely immune to this bias.

Our measured proper motion (117.3 ± 0.2 mas year^{-1} along a position angle of 73.5° east of north) agrees with the value given in the fourth version of the U.S. Naval Observatory CCD Astroglyph Catalog (UCAC4) (18) but is inconsistent with that measured by (5), who found a similar magnitude but a very different position angle. Together with the systemic radial velocity (19), our fitted distance and proper motion imply a peculiar velocity of 55 km s^{-1} relative to the galactic rotation; this value is higher than expected for long-period CVs (20) but still is lower than the 81 km s^{-1} implied by a distance of 159 pc.

Our best-fitting distance of 114 ± 2 pc is in excellent agreement with the distance of ~ 117 pc calculated by (7) for SS Cygni to be compatible with the DIM. As shown in Fig. 2, it brings the mean mass transfer rate down to $2.6 \times 10^{17} \text{ g s}^{-1}$ (7), well below the critical mass transfer rate of $9.0 \times 10^{17} \text{ g s}^{-1}$ (8). Thus, the disc will be unstable, and the DIM can successfully explain the observed semi-regular outbursts. Our fitted distance also brings the peak absolute magnitude of

SS Cygni back in line with that expected for systems of similar orbital period (21).

Our revised distance also tests a second prediction of the DIM: The mass accretion rate at the onset of the outburst decline should be very close to the critical rate. The mass accretion rate predicted by the DIM was approximated by (7) to have a power-law dependence on several key system parameters (the distance, inclination angle, disc size, white dwarf mass, and apparent magnitude). For a distance of 114 pc and the range of system parameters derived by (22), this approximation predicts an apparent visual magnitude in the range 8.8 to 9.5 at the onset of decline, as compared to the observed value of ~ 8.6 . If the DIM is correct, and the approximation of (7) is valid in the revised region of parameter space, this implies that the inclination angle of the system must be close to 45° , and the mass of the white dwarf should be close to the mass of the Sun.

Besides CVs, the DIM has been used to explain the main features of XRB outbursts, when modified to account for the heating effect of x-ray irradiation (23) and the truncation of the inner disc (24). The thermal-viscous instability at the heart of the DIM is also believed to be active in AGN (25), although in that case it only produces relatively small amplitude variability rather than large outbursts (26). In an exact parallel to the distinction between dwarf novae and nova-like CVs, the difference between transient and persistent XRBs is attributed to mass transfer rates either below or above a critical threshold (10, 11). A failure of this model in SS Cygni would there-

fore have called into question our understanding not only of dwarf novae but of transient XRBs.

The controversy over the distance to SS Cygni emphasizes the need for accurate distances to accreting objects. Since they use stationary reference frames, VLBI parallax studies can play a critical role in determining the distance to radio-emitting Galactic sources, especially now that recent sensitivity upgrades to VLBI instruments have enabled the extension of such studies to a larger population of sources.

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Supplementary Materials

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Supplementary Text
Fig. S1
Tables S1 and S2
References (27–33)

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Taxel-Addressable Matrix of Vertical-Nanowire Piezotronic Transistors for Active and Adaptive Tactile Imaging

Wenzhuo Wu,^{1*} Xiaonan Wen,^{1*} Zhong Lin Wang^{1,2†}

Designing, fabricating, and integrating arrays of nanodevices into a functional system are the key to transferring nanoscale science into applicable nanotechnology. We report large-array three-dimensional (3D) circuitry integration of piezotronic transistors based on vertical zinc oxide nanowires as an active taxel-addressable pressure/force sensor matrix for tactile imaging. Using the piezoelectric polarization charges created at a metal-semiconductor interface under strain to gate/modulate the transport process of local charge carriers, we designed independently addressable two-terminal transistor arrays, which convert mechanical stimuli applied to the devices into local electronic controlling signals. The device matrix can achieve shape-adaptive high-resolution tactile imaging and self-powered, multidimensional active sensing. The 3D piezotronic transistor array may have applications in human-electronics interfacing, smart skin, and micro- and nanoelectromechanical systems.

Progress has been achieved in implementing flexible pressure sensors based on arrays of tactile pixels (taxels) for mimicking the tactile sensing capabilities of human skin. In these sensors, electronic components such as traditional planar field-effect transistors (FETs) act as readout elements for detecting pressure-induced property

changes in the pressure-sensitive media (1–6). Their continued improvement depends on minimizing the effect of substrate strain on the performance of the electronic components while increasing the flexibility of the substrate (1–3, 7). This scheme of pressure sensing not only requires complicated system integration of hetero-

geneous components but also lacks direct and active interfacing between electronics and mechanical actuations. Moreover, the sizes of as-fabricated taxels are hundreds of micrometers to tens of millimeters, severely limiting device density and spatial resolution. Although architectures such as three-dimensional (3D) integrated circuits and wrap-gate vertical transistors present attractive approaches to achieving high-density assembly of functional nanodevices (8–11), it is cumbersome to fabricate the gate electrode and manage the interconnect layout so as to effectively control an individual device within a high-density matrix. A schematic of a representative wrap-gate nanowire (NW) FET is shown in Fig. 1A.

Recently, using piezoelectric semiconductor NWs that typically have wurtzite and zinc blend structures (such as ZnO and GaN), a piezotronic transistor design was introduced (12–15) that has a two-terminal metal-semiconductor-metal structure and whose charge carrier transport is modulated by the piezoelectric polarization charge-

¹School of Materials Science and Engineering, Georgia Institute of Technology, Atlanta, GA 30332, USA. ²Beijing Institute of Nanoenergy and Nanosystems, Chinese Academy of Sciences, Beijing 100083, China.

*These authors contributed equally to this work.

†Corresponding author. E-mail: zhong.wang@mse.gatech.edu

induced inner-crystal potential in the NW at the contacts. The piezotronic effect differs from the conventionally used piezoresistive effect in that the latter results from a change in band gap, charge carrier density, or density of states in the conduction band of the strained semiconductor material (which functions as a scalar “resistor”), whereas the piezotronic effect arises as a result of the polarization of nonmobile ions in the crystal. Therefore, the piezoresistive effect is a symmetric volume effect without polarity, whereas the piezotronic effect is an interface effect that asymmetrically modulates local contacts at different terminals of the device because of the polarity of the piezoelectric potential (piezopotential) (15–18). The magnitude and polarity of piezopotential within a piezotronic transistor changes according to the local stress or force, resulting in a direct con-

trol over local Schottky barrier heights (SBHs) and hence the corresponding transport characteristics of the piezotronic transistor by induced strain. Consequently, no applied electrical gate voltage is required for a piezotronic transistor. Strain-gated piezotronic transistors operate through the modulation of local contact characteristics and charge carrier transport by strain-induced ionic polarization charges at the interface of a metal-semiconductor contact (13, 16–20), which is different from the voltage-gated operation of a traditional FET.

The elimination of a wrap gate also offers a new approach for 3D structuring. The basic structure of a 3D strain-gated vertical piezotronic transistor (SGVPT) (Fig. 1A, right) consists of one or more vertically grown ZnO NWs in contact with bottom and top electrodes. Each ZnO NW experiences axial strain when subjected to external

mechanical deformation, with piezopotential induced inside the NW as a result of polarization of nonmobile ions distributed at the two ends (15, 17, 18). The local contact profile and carrier transport characteristics across the Schottky barrier, formed between the ZnO NW and metal electrodes, is effectively controlled by the polarization charge-induced potential. The electrical characteristics of the two-terminal SGVPT are therefore modulated by external mechanical actions induced by strain; that is, strain essentially functions as a gate signal for controlling the SGVPT.

By combining the patterned in-place growth of vertically aligned ZnO NWs with state-of-the-art microfabrication techniques, large-scale integration of a SGVPT array can be obtained. Figure 1B illustrates a 1-cm² SGVPT array with a taxel density of 92 × 92 (234 taxels per inch). The

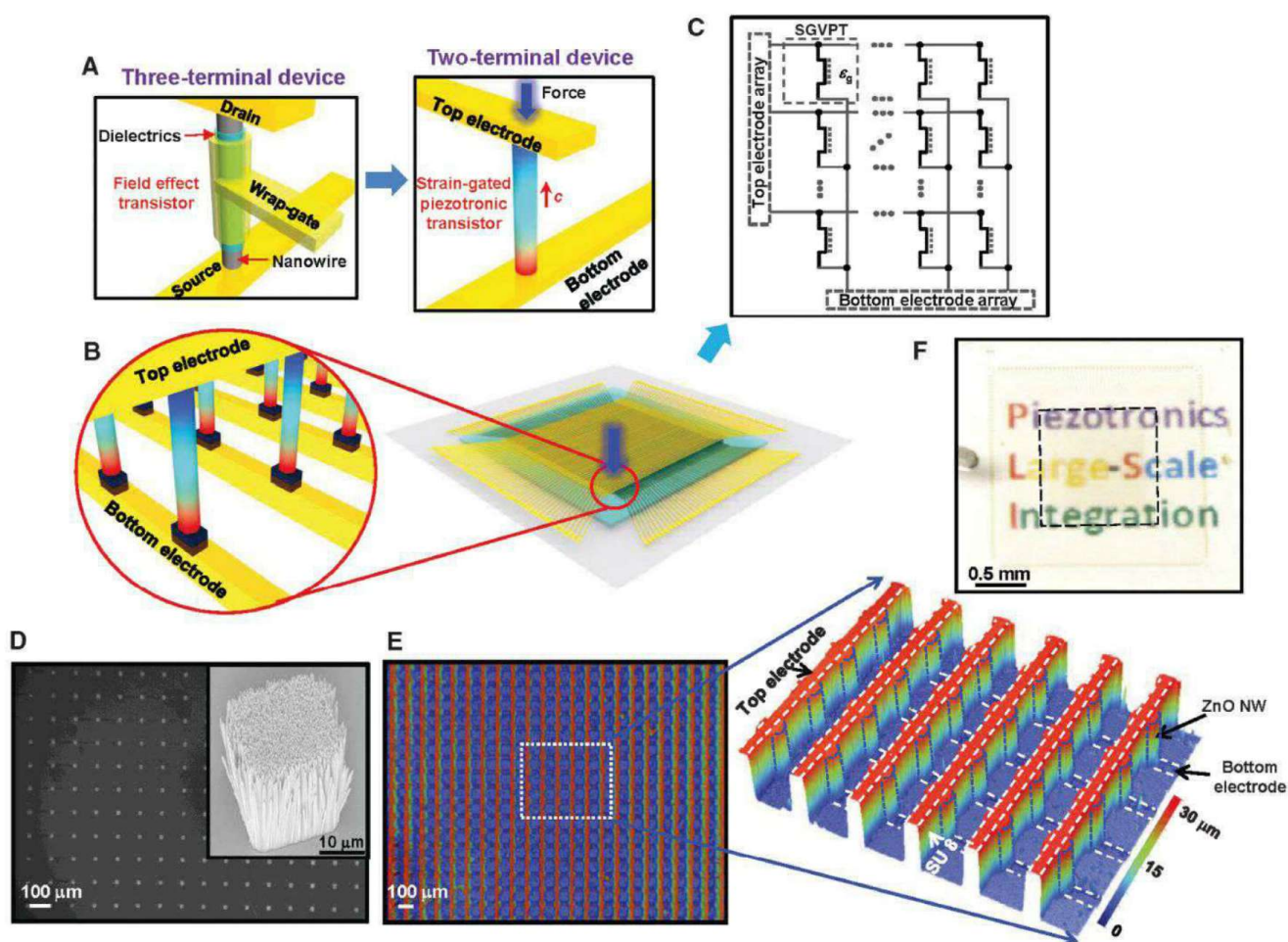


Fig. 1. Schematic illustration, optical and electron microscopic images, and topological profile image of 3D SGVPT array assembly. (A) Comparison between three-terminal voltage-gated NW FET (left) and two-terminal strain-gated vertical piezotronic transistor (right). Color gradient in the strained SGVPT represents the strain-induced piezopotential field, in which red and blue indicate positive and negative piezopotential, respectively. ZnO NWs in SGVPT grow along the c axis (red arrow). (B) Schematic illustration of a 3D SGVPT array with taxel density of 92 × 92 and scheme for spatial profile imaging of local stress (indicated by the downward blue arrow) by the array (zoom-in schematic). (C) Equivalent circuit diagram of the 3D SGVPT array. The region highlighted by black dashed lines is the unit SGVPT device, in which ϵ_g represents the

mechanical strain gate signal and the vertical dotted line between the two terminals of the SGVPT denotes the modulation effect of ϵ_g on the conducting characteristics of the device. (D) Scanning electron micrograph of SGVPT array taken after etching back the SU 8 layer and exposing top portions (~20 μ m) of the ZnO NWs. Inset shows 30°-tilt view of the exposed ZnO NWs for a single taxel. (E) Topological profile image of the SGVPT array (top view). At right, a 3D perspective view of the topological profile image reveals the vertical hierarchy of the SGVPT assembly; the color gradient represents different heights, as indicated. (F) Optical image of the transparent 3D SGVPT array on a flexible substrate. The peripherals are the pads of the device, and the central region highlighted by black dashed lines is the active array of 3D SGVPTs.

equivalent circuit diagram of the SGVPT (Fig. 1C) shows the operation scheme of the SGVPT device circuitry. The taxel area density of the SGVPT array is 8464 cm^{-2} , which is higher than the number of tactile sensors in recent reports (~ 6 to 27 cm^{-2}) (1–3, 6) and mechanoreceptors embedded in human fingertip skin ($\sim 240 \text{ cm}^{-2}$) (21). A detailed description of the device fabrication process is shown in the supplementary materials and figs. S1 and S2. Briefly, the active array of SGVPTs is sandwiched between the top and bottom indium tin oxide (ITO) electrodes, which are aligned in orthogonal cross-bar configurations. A thin layer of Au is deposited between the top and bottom surfaces of ZnO NWs and the top and bottom ITO electrodes, respectively, forming Schottky contacts with ZnO NWs. A thin layer of Parylene C (SCS Labcoater; thickness $1 \mu\text{m}$) is conformably coated on the SGVPT device as the

moisture and corrosion barrier. Well-aligned ZnO NWs, synthesized by a low-temperature hydrothermal method (22), function as the active channel material of the SGVPT and help reduce the stochastic taxel-to-taxel variation to ensure uniform device performance. Figure 1D and fig. S3 show the SGVPT array after etching back the encapsulation SU 8 layer (epoxy-based negative photoresist) and exposing the top portions ($\sim 20 \mu\text{m}$) of the ZnO NWs (see supplementary materials for fabrication details). The as-synthesized ZnO NWs show single crystallinity (fig. S3).

The 3D nature and vertical hierarchy of the SGVPT assembly is revealed by topological profile imaging (Fig. 1E) using an optical noncontact profilometer (Wyko Profilometer NT3300), which measures the phase change of light reflected from various heights in the structure by interferometry. The high degree of alignment and uniformity of

the SGVPT array in three dimensions ($\sim 30 \mu\text{m}$ in height, $20 \mu\text{m} \times 20 \mu\text{m}$ in taxel size) is enabled by process control in both the bottom-up synthesis of NWs and the top-down fabrication of circuitry. The use of a two-terminal configuration based on the piezotronic effect simplifies the layout design and circuitry fabrication while maintaining effective control over individual devices. The transparency and flexibility of SGVPT array devices are shown in Fig. 1F and figs. S4 and S5.

By selecting one top electrode, the 92 taxels between this top electrode and the corresponding bottom electrodes can be addressed and characterized individually (see supplementary materials). Representative data from 23 taxels in a typical single-channel line scan (1×92) measurement for a SGVPT array device are shown in Fig. 2. The corresponding topological profile images (top view) of the selected taxels are displayed at

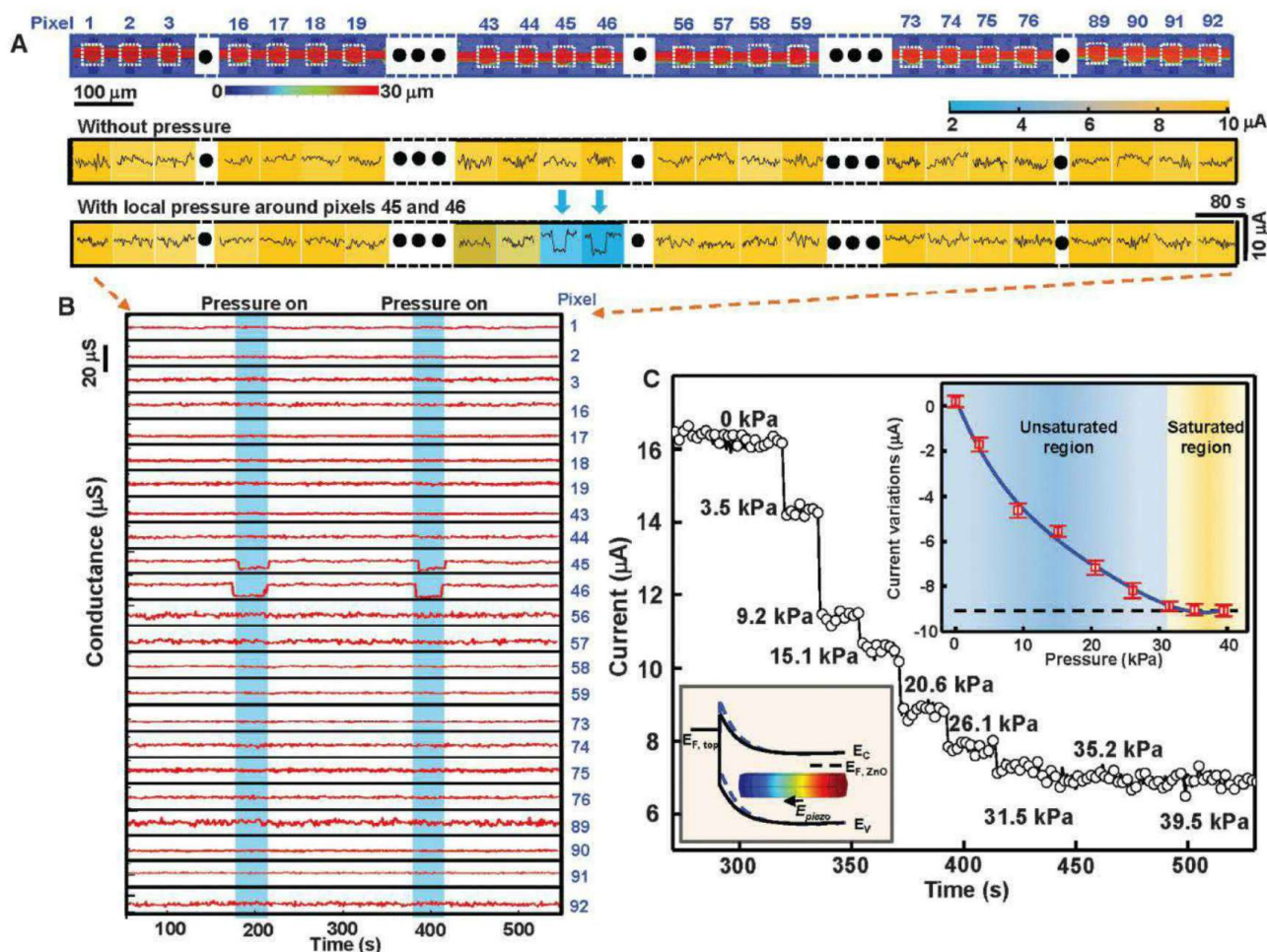


Fig. 2. Single-channel line scan (1×92) electrical measurement for SGVPT array device. (A) Topological profile images (top view) of 23 selected taxels in a 1×92 SGVPT array (single channel; top frame) and their corresponding current responses (middle and bottom frames) under 1 V bias without and with external stress (20 kPa) applied to a localized region around taxels 45 and 46. (B) Single-channel conductance measurement in the temporal domain, illustrating the dynamic response of the 23 selected SGVPT devices in this channel, without and with pressure applied. (C) Current responses for taxel 46 under different pressures, showing the gate modulation effect of applied pressure on the electrical characteristics of the SGVPT. Top right inset:

Current variations (red squares) are plotted versus the applied pressures, clearly showing the saturation of current change when applied pressure is above ~ 30 kPa. Bottom left inset: Schematic band diagram illustrating the change in SBH of the reverse-biased top contact due to the modulation effect of strain-induced piezopotential. Color gradient represents the distribution of piezopotential field (red indicates positive piezopotential; blue indicates negative piezopotential). The original band edges at the reverse-biased Schottky contact for the SGVPT device without stress applied are shown as black solid lines. The band edges bending at the reverse-biased Schottky contact for the SGVPT device with stress applied are shown as blue dashed lines.

the top of Fig. 2A. The current response from each taxel under 1 V bias, with and without external pressure (20 kPa) applied to a localized region (around taxels 45 and 46), is recorded and plotted, with colors representing the ratio of the response amplitude for each taxel in an 80-s window. It can be seen that for this single-channel array of SGVPTs, pressure variations can be distinguished with both high sensitivity and spatial resolution (taxel periodicity, $\sim 100\ \mu\text{m}$). The dominant mechanism for the transport property of SGVPT is the piezotronic effect rather than the piezoresistance effect, as experimentally confirmed and elaborated (fig. S6) (15, 17, 18). Data from single-channel conductance measurements in the temporal domain are compiled and shown in Fig. 2B and fig. S7 to further illustrate the dynamic response of SGVPT devices. Distinctive changes in conductance can be observed for taxels 45 and 46 before and after applying the localized pressure. Although the measured response time (rise time) of $\sim 0.15\ \text{s}$ for a SGVPT taxel (fig. S7) is larger than that of human fingertips (~ 30 to $50\ \text{ms}$), it is comparable to previously reported values of $0.1\ \text{s}$ (1). These results indicate that a SGVPT array can respond to static

as well as dynamic stimuli. The response time can be further improved in future designs by integrating local on-site signal processing circuits with a SGVPT array (23).

The pressure sensitivity of a single SGVPT is shown in Fig. 2C for taxel 46. We applied increasing pressure at a fixed location on the SGVPT and measured the variations in current response. The SGVPT device demonstrated high sensitivity for detecting pressure change, particularly in low-pressure regions ($<10\ \text{kPa}$). A plot of current variation versus pressure change (Fig. 2C, top right inset) shows the modulation effect of applied pressure. It can be seen that the maximum pressure at which a SGVPT taxel can still distinguish without “saturation” is around 30 kPa, above which the current saturates. The observed sensing range of a few kPa to $\sim 30\ \text{kPa}$ for a SGVPT array is well matched to the range of pressure that a human finger applies to sense texture and shape, 10 to 40 kPa (24). The sensitivity of a SGVPT, defined as $S = dG_{\text{SGVPT}}/dP$ (where G is the measured conductance for SGVPT), is around $2.1\ \mu\text{S kPa}^{-1}$; this value arises from the change in carrier transport of the SGVPT by applied pressure due to the corre-

sponding modulation of barrier height at the reverse-biased Schottky contact by strain-induced piezopotential (15, 17, 18).

The conductance of a SGVPT device is dictated by the reverse-biased Schottky contact, which is formed between ZnO NWs and top electrodes in this case. Upon applying the normal stress, accumulation of piezoelectric charges at both Schottky contacts induces the distribution of piezopotential. Because of the orientation of the polar c axis in the as-synthesized ZnO NWs (red arrow in Fig. 1A, right panel), negative piezopotential is induced at the reverse-biased top Schottky contact, which raises the barrier height at that contact and hence decreases the transport conductance of the SGVPT taxel, as depicted by the schematic band diagrams in Fig. 2C (bottom left inset) and fig. S6. The operation of the SGVPT device is therefore based on barrier-interface modulation that enables enhanced sensitivity and efficiency relative to the channel modulation operation in conventional FETs. The quality of the Schottky contacts has been characterized (supplementary materials and fig. S8). The SBHs and ideality factors of the formed contacts for devices without extra oxygen plasma treatment before depositing the top electrode are $0.419 \pm 0.011\ \text{eV}$ and 5.84 ± 1.29 , respectively, whereas the SBHs and ideality factors of the formed contacts for devices with extra oxygen plasma treatment before depositing the top electrode are found to be $0.575 \pm 0.013\ \text{eV}$ and 2.17 ± 0.33 , respectively. These results indicate that the qualities of as-fabricated Schottky contacts have been improved by the oxygen plasma treatment (19).

The feasibility and scalability of the proposed integration scheme are demonstrated by the successful fabrication of the 92×92 -taxel SGVPT array, enabling a factor of 15 to 25 increase in number of taxels and a factor of 300 to 1000 increase in taxel area density relative to recent reports (1–3, 6). The output current of each individual SGVPT taxel is measured and averaged within a short duration window of 10 ms. By monitoring the output current of each independently functioning SGVPT in the matrix, a spatial profile of applied pressure can be readily imaged by multiplexed addressing of all the taxels. A 2D current contour plot was thus obtained by registering the measured current to the corresponding taxel coordinates along the x (bottom electrode) and y (top electrode) axes. Metrology mapping was then performed on the fully integrated SGVPT array without applying pressure (Fig. 3A, inset), demonstrating that all of the 8464 SGVPTs within the array are functional. Subsequent statistical investigation revealed good uniformity in electrical characteristics among all taxels, with 95% of the SGVPTs possessing current values in the narrow range of $13.7 \pm 2.73\ \mu\text{A}$ under 1 V bias (Fig. 3A). The uniformity in the current distribution of SGVPTs can be further improved by optimizing the fabrication process, such as achieving a uniform number of ZnO NWs within each taxel (fig. S3) and obtaining even profiles in the etch-back step of

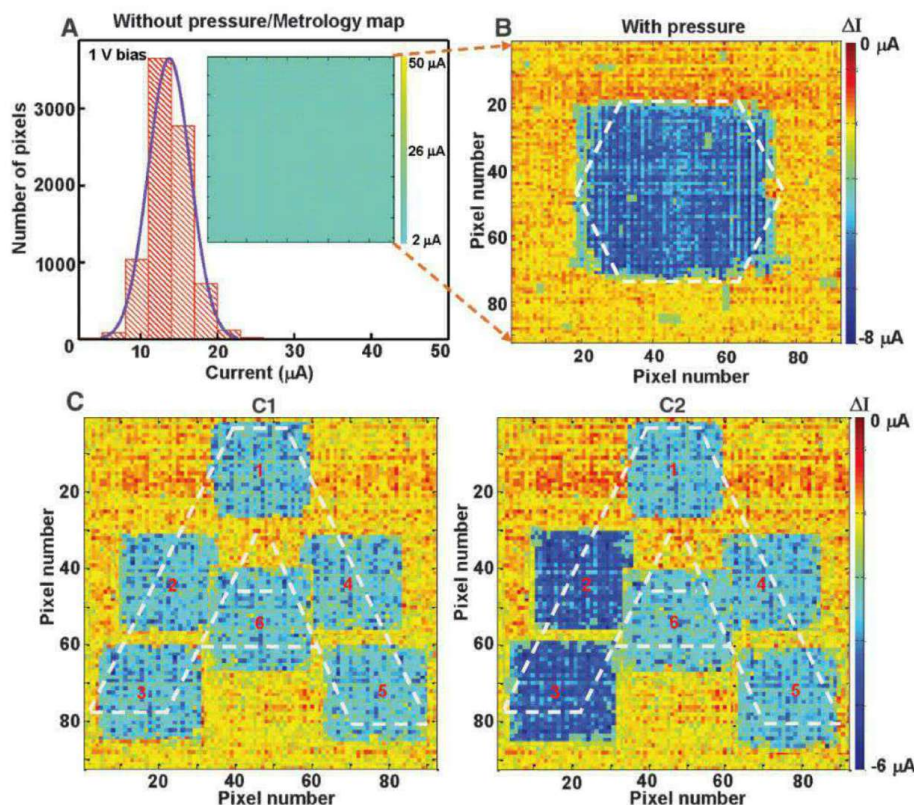


Fig. 3. Tactile imaging and multidimensional sensing by the fully integrated 92×92 SGVPT array. (A) Metrology mapping (inset) and statistical investigation of the fully integrated SGVPT array without applying stress. (B) Current response contour plot illustrating the capability of SGVPT array for imaging the spatial profile of applied stress. Color scale represents the current differences for each taxel before and after applying the normal stress. The physical shape of the applied stress is highlighted by the white dashed lines. (C) Multidimensional sensing by an SGVPT array exhibits the potential of realizing applications such as personal signature recognition with maximum security and unique identity. The shape of a “written” letter A is highlighted by the white dashed lines.

the SU 8 layer. To demonstrate the tactile sensing capability of the integrated SGVPT array, we applied a normal stress of ~ 25 kPa to the device by pressing a hexagonal mold. As shown in Fig. 3B, which presents for each taxel the difference between current values before and after applying the normal stress, the profile of applied stress can be spatially imaged.

The SGVPT devices remained operational and capable of imaging the spatial profile of applied pressure after 24 hours of immersion in 23°C deionized water and 37°C 0.9% saline solution, as well as after 6 hours of immersion in 65°C deionized water and 65°C 0.9% saline solution (figs. S9 and S10), indicating good stability and feasibility of SGVPT array operation for future applications such as in vivo physiological sensing in complex environments. Deteriorated adhesion between the encapsulation SU 8 layer in the SGVPT array and the substrate was observed for devices after prolonged immersion (12 hours) in both solutions at 65°C. Note that even in such cases, the top electrodes and taxels remained in good shape; only rupture of the bottom electrodes can be seen (fig. S10).

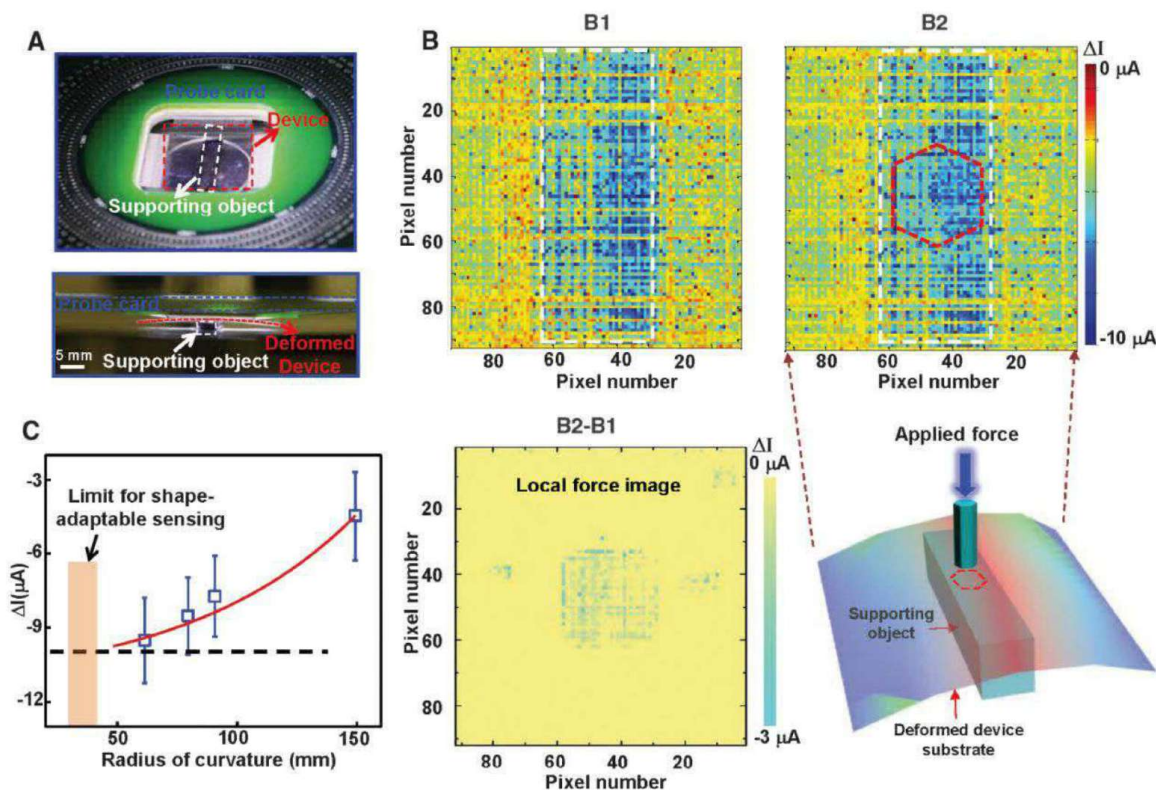
To investigate whether a single device array could resolve the stress profile spatially while also registering the stress variations to the mapped geometrical pattern (as enabled by the uniform sensitive response of taxels across the whole array and the high spatial taxel density), we used a SGVPT device (Fig. 3C) fabricated on a solid

silicon substrate. A three-axis stage and force gauge was used to apply normal stresses with well-determined values and spatial locations to the device (see supplementary materials). The first group of six normal local stresses, each around 8.1 kPa, were applied to the array at preprogrammed locations in a sequence indexed from 1 to 6, with the corresponding contours imaged and organized (Fig. 3C, panel C1) to emulate the process of writing the letter A. This process was achieved by varying the x and y coordinates while keeping the z coordinate constant in the control interface of the three-axis stage. It can be seen from Fig. 3C (panel C1) that spatial profiles of all six applied stresses can be distinguished and mapped electronically. The second group of six normal stresses with the same locations were then applied to the array in the same sequence, except that the stresses applied at sites 2 and 3 were increased to ~ 20 kPa; stresses at the other four sites were unchanged. The corresponding mapped contours were again recorded and organized (Fig. 3C, panel C2). These results demonstrate the potential of using an SGVPT array for future applications such as multidimensional signature recording, which not only records the calligraphy or signature patterns when people write, but also registers the corresponding pressure or force applied at each location (dictated by the resolution of the taxel array) by the writer. This augmented capability enables personal signature recognition with unique identity and enhanced security.

The real-time detection of shape changes caused by stretching or twisting is a desirable feature for sensors embedded in an artificial tissue or prosthetic device. Figure 4A shows the experimental setup for investigating the feasibility of a SGVPT array for shape-adaptive sensing. A rectangular supporting object is affixed to the platen of probe station, directly beneath the central region of the SGVPT array. After the probe pins are in contact with the pads at the periphery of the SGVPT device, the platen is further raised up so that the device is bent by the supporting object beneath (Fig. 4A, bottom) with a radius of curvature of ~ 79.63 mm. The plot of measured difference in taxel currents with and without the supporting object (Fig. 4B, panel B1) illustrates a good agreement between the detected shape change of the SGVPT array and the physical shape of the supporting object beneath. The shape-adaptive sensing capability was further examined by applying an additional localized stress to the bent SGVPT array, using the same setup in Fig. 3, B and C, as depicted by the 3D schematic drawing in Fig. 4B (bottom right). The measured variation in taxel current values between the bent SGVPT array with extra stress and the unstrained SGVPT array is shown in Fig. 4B (panel B2). A clearer demonstration of the data can be obtained by numerical subtraction (Fig. 4B, panel B2–B1), which gives rise to spatial imaging of the additionally applied stress when the shape of the SGVPT device changes. Such shape-adaptive sensing has

Fig. 4. Shape-adaptive sensing by the flexible 92×92 SGVPT array.

(A) Photographs of the experimental setup for investigating the feasibility of a SGVPT array for shape-adaptive sensing. Upper image, top view; lower image, side view with the device deformed. (B) Shape-adaptive sensing of the SGVPT array. (B1) The measured difference in taxel currents for the SGVPT array with and without a supporting object beneath. The detected shape change of the SGVPT array is illustrated by the dark blue regions; the physical shape of the supporting object beneath the SGVPT device is outlined by the white dashed lines. (B2) The measured variations in taxel current values between bent the SGVPT array with extra stress and the unstrained SGVPT array. The location of the extra stress is outlined by the red dashed lines. (B2–B1) A clearer demonstration of the data is obtained by numerically subtracting B1 from B2, giving rise to spatial imaging of the additionally applied stress



when the shape of the SGVPT device is changed. The 3D schematic drawing at the lower right illustrates the process for shape-adaptive sensing. (C) Experimental results show the limit for shape-adaptable sensing by a SGVPT array with the present design.

also been investigated for other radii of curvature (fig. S11). Because of the relatively large thickness of the SGVPT device (mainly contributed by the polyethylene terephthalate substrate, which is 500 μm thick), the saturation of SGVPT response under high pressure (as shown in Fig. 2C), and the constraints of the measurement setup (e.g., limited vertical movement of probes), the SGVPT array is unable to sense the change in device shape and further distinguish the applied pressure when the radius of curvature is smaller than 30 to 35 mm (Fig. 4C). The detectable range of shape deformation (and of the corresponding shape-adaptive sensing) can be improved by engineering the device into more compliant form to reduce the strain induced in the SGVPT as a result of changes in device shape (fig. S12).

A SGVPT array was repeatedly bent to a very small radius of curvature (15 mm, as shown in fig. S13) at a frequency of 2 Hz to simulate accelerated aging. Metrology mapping was then performed on the array and plotted for comparison with that of the device before the cyclic bending. No obvious degradation in SGVPT array operation could be observed even after 1000 cycles of

bending, suggesting good reliability and stability in device operation.

The SGVPT devices described above can function as active and self-powered tactile sensors by directly converting applied mechanical actuations into electrical control signals without applying gate voltage (fig. S14). Hence, they can act as a fundamental component for piezotronics (15, 25).

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Supplementary Materials

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Figs. S1 to S16

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Periodic Segregation of Solute Atoms in Fully Coherent Twin Boundaries

J. F. Nie,^{1*} Y. M. Zhu,¹ J. Z. Liu,² X. Y. Fang³

The formability and mechanical properties of many engineering alloys are intimately related to the formation and growth of twins. Understanding the structure and chemistry of twin boundaries at the atomic scale is crucial if we are to properly tailor twins to achieve a new range of desired properties. We report an unusual phenomenon in magnesium alloys that until now was thought unlikely: the equilibrium segregation of solute atoms into patterns within fully coherent terraces of deformation twin boundaries. This ordered segregation provides a pinning effect for twin boundaries, leading to a concomitant but unusual situation in which annealing strengthens rather than weakens these alloys. The findings point to a platform for engineering nano-twinned structures through solute atoms. This may lead to new alloy compositions and thermomechanical processes.

Interfaces such as twin and grain boundaries play a critical role in plastic deformation and ultimately in controlling the formability and mechanical properties of many engineering materials (1–5); notable examples are lightweight magnesium (Mg) alloys, which have received considerable attention for applications leading to fuel efficiency and green environment (6). Like other commonly used metals such as titanium (Ti), zirconium (Zr), and zinc (Zn), Mg has a hexagonal structure with fewer slip systems than those of cubic materials. To readily form Mg products requires the activation of

twinning modes for plastic deformation. As an emerging class of engineering materials, Mg alloys are less strong than the counterpart aluminum alloys, implying the need for more efficient barriers in order to impede the motion of dislocations and twin boundaries. The control of deformation twinning during thermomechanical processes and applications is a major technical barrier to the wider application of Mg (7). Twinning occurs predominantly in the $\langle 10\bar{1}1 \rangle \{10\bar{1}2\}$ system (where $\{10\bar{1}2\}$ is the twinning plane and $\langle 10\bar{1}1 \rangle$ is the twinning direction in the twinning plane), although $\langle 10\bar{1}2 \rangle \{10\bar{1}1\}$ and $\langle 3032 \rangle \{10\bar{1}3\}$ have also been observed (8–13). The formability, yield strength, and tension–compression yield-strength asymmetry of wrought Mg products are all intimately related to twinning; hence, there have been considerable efforts to gain a fundamental understanding of the nucleation, growth, and thermal stability of such deformation twins and of

the factors that dictate their development under different loading conditions. However, gaining fundamental insights from experimental observations of the effects of solute, second-phase particles, grain size, and sample size on deformation twinning in Mg (14–16) has proved elusive. This has also been the case more broadly in engineering materials (17–19). Specifically, we need atomic-scale experimental evidence and an understanding of the structure and chemistry of twin boundaries in alloys.

In contrast with partially coherent interfaces such as high-angle grain boundaries and symmetrical tilt boundaries with arrays of misfit dislocations, for which segregation of alloying elements is well established (20–22), fully coherent twin boundaries have low interfacial energies, and solute segregation in such boundaries is therefore not expected. We studied this issue using high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) to observe the migration and segregation of randomly distributed solute atoms to fully coherent terraces of deformation twin boundaries in Mg alloys. Solute consisted of gadolinium (Gd) (which is larger than Mg and represents rare-earth elements that are major alloying additions in many commercial Mg alloys), Zn (which is smaller than Mg and a major alloying element in some commonly used Mg alloys), and mixtures of Gd and Zn (table S1). We also analyzed the experimental observations using first-principles calculations (fig. S1) and continuum estimates and the impact of the solute segregation on mechanical properties using compression tests. Alloy compositions, the preparation and testing conditions and characterizations, and the details of the computations are available in the supplementary materials.

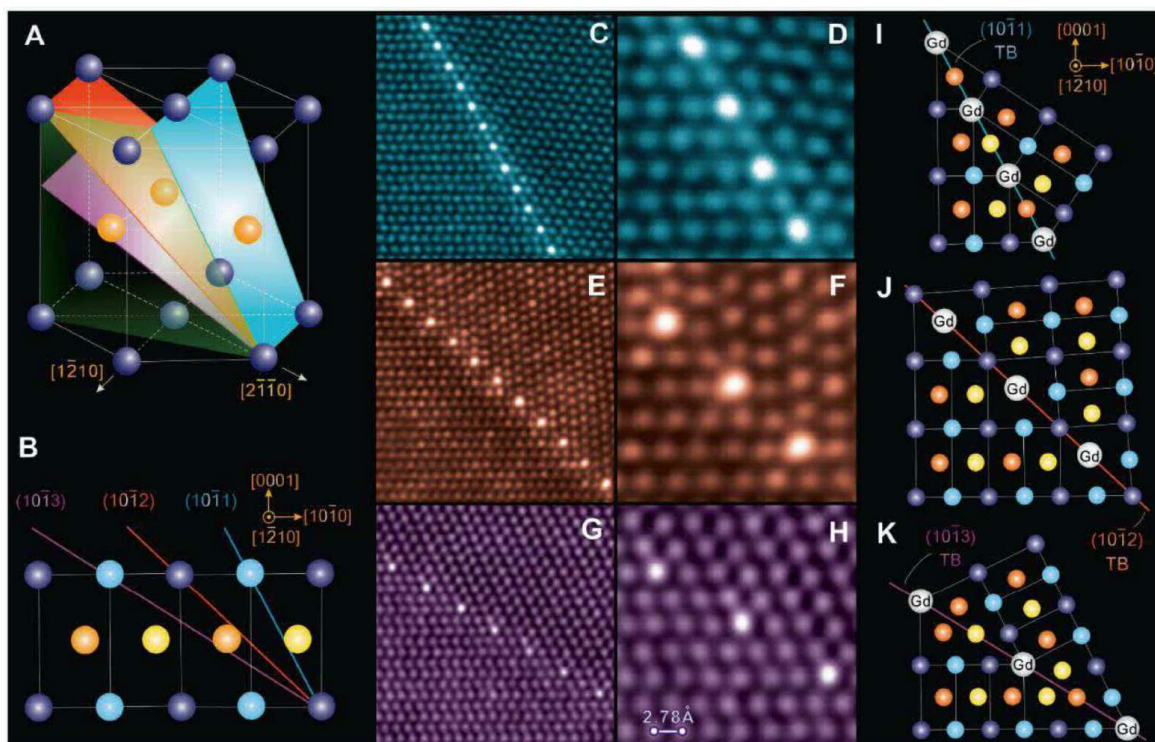
¹Department of Materials Engineering, Monash University, Victoria 3800, Australia. ²Department of Mechanical and Aerospace Engineering, Monash University, Victoria 3800, Australia.

³Monash Centre for Electron Microscopy, Monash University, Victoria 3800, Australia.

*Corresponding author. E-mail: jianfeng.nie@monash.edu

Fig. 1. Periodic segregation of Gd in various twin boundaries (TBs).

(A) Schematic illustration and (B) $[1\bar{2}10]$ -perspective view of α -Mg lattice. The $\{10\bar{1}1\}$, $\{10\bar{1}2\}$, and $\{10\bar{1}3\}$ planes are blue, red, and purple, respectively. (C, E, and G) HAADF-STEM images showing $\{10\bar{1}1\}$, $\{10\bar{1}2\}$, and $\{10\bar{1}3\}$ twin boundaries (TBs) in Mg–0.2 atomic % Gd [(C) and (G)] and Mg–0.8 atomic % Gd (E) solid solution alloys. (D, F, and H) Close-ups of (C), (E), and (G), schematically illustrated in (I to K). In (B) and (I) to (K), atoms in the A layer are blue (in the paper plane) or purple (out of the paper plane) and yellow (in) or orange (out) in the B layer. Sample details are available in table S1.



Microstructural examination of the deformed samples of all alloys indicated the existence of many twins—generally $\{10\bar{1}2\}$, but $\{10\bar{1}1\}$ and $\{10\bar{1}3\}$ were also present in samples subjected to larger compression strains. Inspection of HAADF-STEM images in $\langle 1\bar{2}10 \rangle$ suggested that the boundary terraces were free of apparent solute segregation (fig. S2). However, twin boundaries in samples that have been plastically compressed and subsequently heat-treated were quite different (fig. S3). Shown in Fig. 1 are the atomic-resolution HAADF-STEM images of retained $\{10\bar{1}1\}$, $\{10\bar{1}2\}$, and $\{10\bar{1}3\}$ twin boundaries in two binary Mg–Gd alloys that have been plastically compressed at room temperature and annealed for either 5 or 60 min at 275°C. We found that all twin boundary images were decorated by a periodic distribution of bright dots. Because the brightness of individual columns of atoms in HAADF-STEM approximates the square of Z (23, 24)—the averaged atomic number—each bright dot represents a column rich in Gd atoms. The Gd-rich and Mg columns were distributed alternately along the boundaries, with a Mg column between the two adjacent Gd-rich columns. Careful examination of the HAADF-STEM images (Fig. 1, C, E, and G) indicated that the Gd-rich columns were invariably located in the apparently dilated sites of the twin boundaries, irrespective of the type of twin—that is, whether the twin was $\{10\bar{1}1\}$, $\{10\bar{1}2\}$, or $\{10\bar{1}3\}$.

Shown in Fig. 2 are $\{10\bar{1}2\}$ twin boundaries in a Mg–Zn binary alloy and a Mg–Gd–Zn ternary alloy. Again, a periodic segregation of solute atoms was visible in the twin boundaries. However, in the Mg–Zn alloy, Zn atoms occupied the

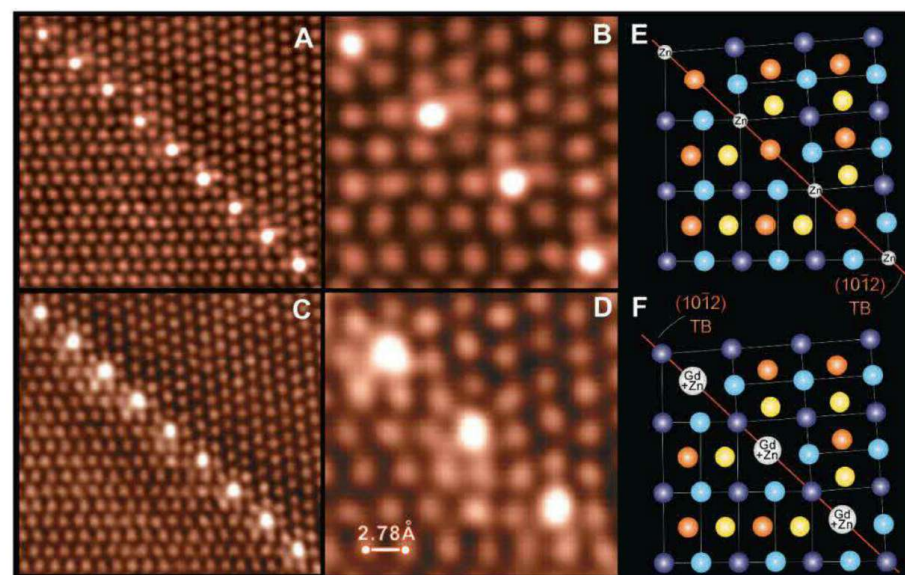


Fig. 2. Periodic segregation of solutes in TBs. HAADF-STEM images showing $\{10\bar{1}2\}$ TBs in (A and B) Mg–1.9 atomic % Zn and (C and D) Mg–1.0 atomic % Gd–0.4 atomic % Zn–0.2 atomic % Zr alloys. (E) and (F) are schematic illustrations of (B) and (D). Sample details are available in table S1.

compressed sites of the twin boundary (Fig. 2E), whereas in the Mg–Gd–Zn alloy, the Gd and Zn atoms took the extension sites (Fig. 2F). In the Mg–Gd–Zn alloy, energy-dispersive x-ray spectra such as the one in fig. S4 indicated an enrichment of Gd and Zn within the $\{10\bar{1}2\}$ boundary, and the absence of bright dots at the compressed sites of the boundary suggested that Zn atoms had most likely joined the Gd atoms to occupy the extended sites of the boundary, in contrast with the Zn atoms' occupancy of compressed sites in the Mg–Zn alloy.

The in-plane atomic local strain hydrostatic invariant (ALSHI) in $\{10\bar{1}1\}$, $\{10\bar{1}2\}$, and $\{10\bar{1}3\}$ twin boundaries of pure Mg are shown in Fig. 3, A to C, and were obtained by means of density functional theory (DFT) computations. We observed periodic distribution of extension (positive) and compression (negative) strains in each case. The strains in the $\{10\bar{1}2\}$ boundary were reduced (Fig. 3, D to F) after the periodic segregation of Gd atoms into the extension sites (Fig. 1J), of Zn atoms into the compression sites (Fig. 2E), or of both Gd and Zn atoms into the

Fig. 3. Strain energy minimization induced periodic segregation of solute atoms in TBs. (A to C) In-plane ALSHI in $\{10\bar{1}1\}$, $\{10\bar{1}2\}$, and $\{10\bar{1}3\}$ TBs of pure Mg, respectively, and in $\{10\bar{1}2\}$ after segregation of (D) Gd into extension sites, (E) Zn into compression sites, and (F) Gd and Zn into extension sites. The color strip at the left shows the strain magnitude. (G) System total energy reduction with solute concentration in a $\{10\bar{1}2\}$ boundary. (H and I) ALSHI in $\{10\bar{1}2\}$ boundary with Gd in the extension sites, and with Zn segregated into (H) extension and (I) compression sites. (J) System total energy reduction with various Gd and Zn segregations in $\{10\bar{1}2\}$ boundary. The viewing direction is parallel to $\langle 12\bar{1}0 \rangle$ for (A) to (F) and perpendicular to $\{10\bar{1}2\}$ for (H) and (I). ΔE_A and ΔE_V represent the reductions in system total energy normalized by TB area and volume, respectively. A detailed explanation is available in the supplementary materials.

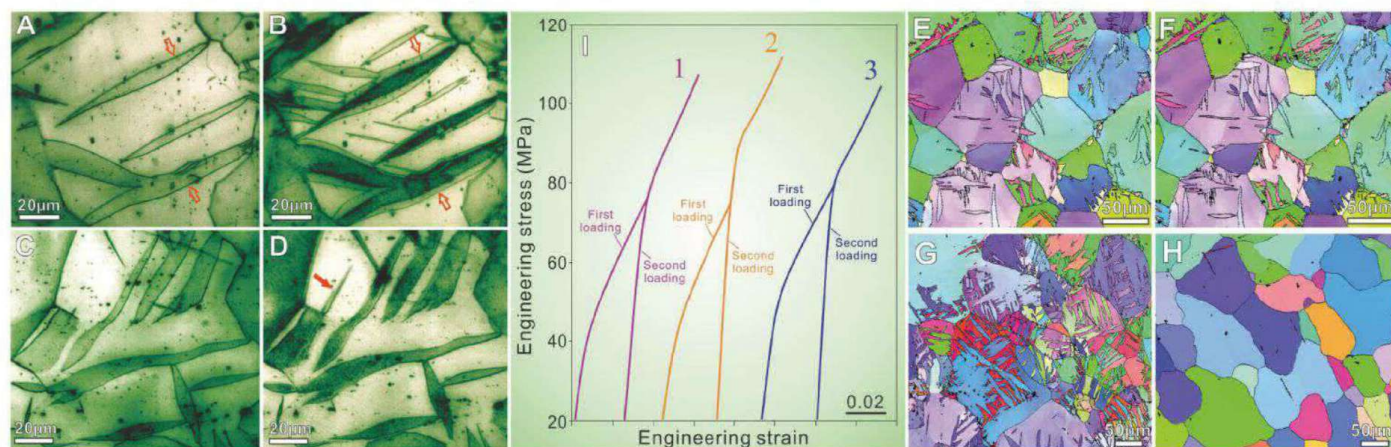
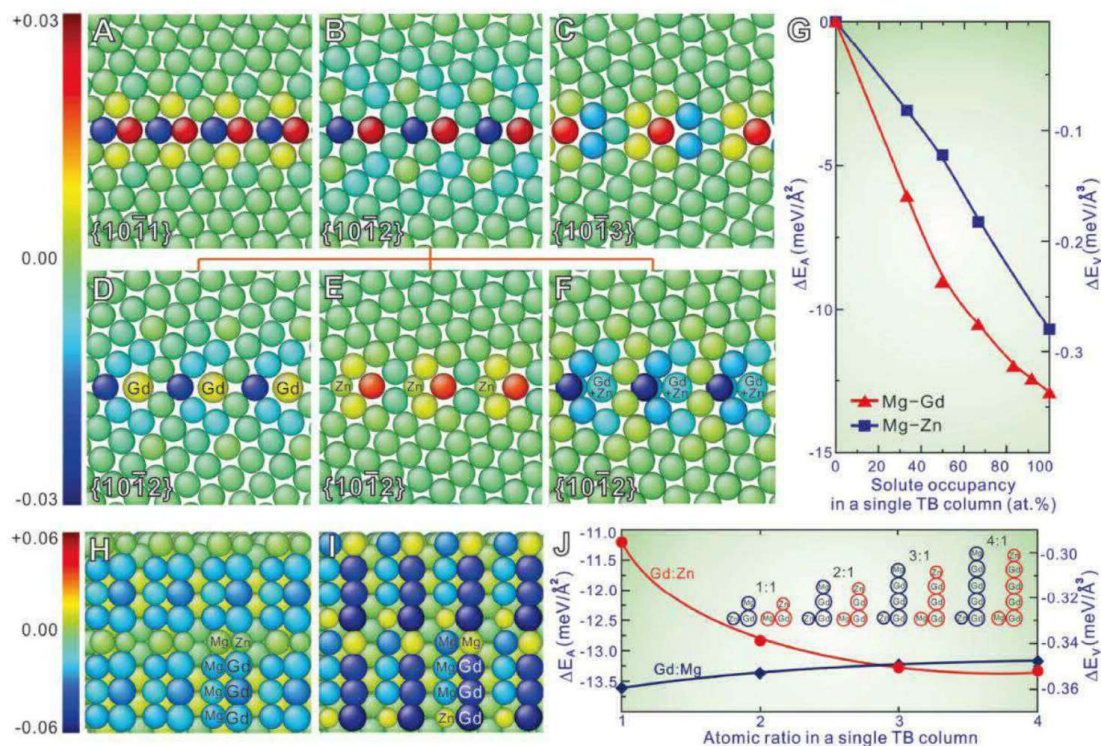


Fig. 4. Pinning effect on TBs and annealing strengthening. (A to D) Optical micrographs showing twins in Mg–0.2 atomic % Gd alloy. (A) Sample compressed to a strain of 0.025, and (B) unloaded and immediately recompressed to an accumulated strain of 0.045. (C) Sample compressed to a strain of 0.025 and (D) unloaded, immediately annealed at 150°C for 3 hours, and recompressed to an accumulated strain of 0.045. (E to H) Electron back-scatter diffraction (EBSD) maps showing microstructure changes. (E) Mg–0.2 atomic % Gd alloy compressed to 0.080 strain, and (F) unloaded, annealed at 300°C

for 20 min. (G) Mg–0.4 atomic % Zn alloy compressed to 0.080 strain, and (H) unloaded, annealed at 300°C for 20 min. For each sample, EBSD maps were obtained from exactly the same location after the heat treatment. (I) Engineering stress–strain curves of three samples of the same alloy from compression tests. Curve 1 is the same test as for (A) and (B). Curve 2 is the same test as for (C) and (D). Curve 3 is the same as for curve 1 but heat treated at 150°C for 3 hours before first loading. Sample details are available in table S1.

extension sites (Fig. 2F). The segregation of Gd, Zn, or both significantly reduces the elastic strain of the twin boundary. The atomic radii of Gd, Zn, and Mg are 0.180, 0.133, and 0.160 nm, respectively. Substituting a Mg atom with a Gd atom leads to a large negative misfit (–0.125) and compression strain, whereas substitution with Zn causes a slightly larger but positive misfit (0.169) and extension strain. Therefore, Gd or Zn atoms in

the Mg solid solution tended to segregate into twin boundaries to minimize the elastic strains associated with individual Gd or Zn atoms and different types of twin boundaries, which in turn reduced the twin boundary energy and the total energy of the system (Fig. 3G, figs. S5 and S6, and table S2). When both Gd and Zn atoms were available, the segregation of Zn atoms into Gd-rich columns led to a greater reduction in system

energy, particularly when each Gd-rich column has more than three consecutively packed Gd atoms (Fig. 3, H to J, fig. S7, and table S3).

Thermodynamically, the segregation or aggregation of Gd or Zn atoms (or both) is not expected to occur within the Mg solid solution, particularly when the concentration of added solute atoms is below the equilibrium solid solubility at the annealing temperature. However, when

elastic strains associated with twin boundaries were introduced into the solid solution matrix, the segregation of Gd or Zn atoms, or both, into the strained sites led to reduced elastic strain energies associated with Gd or Zn atoms and twin boundaries. The DFT computations and continuum estimates both indicated that the ordered segregation of solute atoms in twin boundaries was driven by the minimization of the total energy (fig. S5) and elastic strain energy (table S4) in the system and did indeed reach equilibrium. These periodic solute segregation patterns can be considered as grain boundary “complexions” (4, 25, 26): They are thermodynamically stable only in twin boundaries.

We examined the effects of this phenomenon on the mobility of twin boundaries in and the deformation behavior of Mg alloys, finding that the ordered distribution of solute atoms exerted a stronger pinning effect on any further migration of the twin boundary than expected for individual solute atoms and, hence, a larger strengthening effect. Experimentally, we studied the pinning effect with two identical specimens of a Mg–Gd solid solution alloy. Both samples were unloaded immediately after compressed to 0.025 strain. After unloading, one specimen was compressed again to an accumulated strain of 0.045, whereas the other was annealed at 150°C for 3 hours and compressed again to an accumulated strain of 0.045. We observed twins in both samples after the first compression (Fig. 4, A and C). For the sample without annealing, we detected further growth of twins generated during the first compression (Fig. 4B, red arrow). The boundaries of most twins were noticeably expanded by the second compression. For the annealed specimen,

the size and shape of most preexisting twins remained almost unchanged (Fig. 4, C and D), but some new, small-sized twins also formed (Fig. 4D, red arrow). Analogous experiments on a Mg–0.4 atomic % Zn solid solution alloy yielded a similar but less thermally stable pinning effect (Fig. 4, E to H, and fig. S8).

The pinning effect on the mechanical properties of a binary Mg–Gd solid solution alloy is shown in Fig. 4I. Three identical samples were compression tested at room temperature. The first sample was loaded, unloaded, and immediately reloaded; we observed little strength change from the testing interruption. The second specimen was annealed at 150°C for 3 hours after unloading and before reloading, which led to an appreciable strengthening effect, rather than the weakening that would be expected according to conventional understanding. Before the first loading of the third specimen, it was given the same heat treatment, but this caused neither a weakening nor the strengthening that was observed in the second specimen.

The findings are expected to lead to new insights into the structure and chemistry of fully coherent twin boundaries in other hexagonal materials and cubic materials, as well as strategies for engineering the design of alloy compositions and thermomechanical processes in order to achieve desired formability and mechanical properties.

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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 to S8
Tables S1 to S4
References (27–46)

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Separation of Hexane Isomers in a Metal-Organic Framework with Triangular Channels

Zoey R. Herm,¹ Brian M. Wiers,¹ Jarad A. Mason,¹ Jasper M. van Baten,² Matthew R. Hudson,³ Pawel Zajdel,⁴ Craig M. Brown,^{3,5} Norberto Masciocchi,⁶ Rajamani Krishna,^{2*} Jeffrey R. Long^{1*}

Metal-organic frameworks can offer pore geometries that are not available in zeolites or other porous media, facilitating distinct types of shape-based molecular separations. Here, we report Fe₂(BDP)₃ (BDP^{2−} = 1,4-benzenedipyrazolate), a highly stable framework with triangular channels that effect the separation of hexane isomers according to the degree of branching. Consistent with the varying abilities of the isomers to wedge along the triangular corners of the structure, adsorption isotherms and calculated isosteric heats indicate an adsorption selectivity order of *n*-hexane > 2-methylpentane > 3-methylpentane > 2,3-dimethylbutane ≈ 2,2-dimethylbutane. A breakthrough experiment performed at 160°C with an equimolar mixture of all five molecules confirms that the dibranched isomers elute first from a bed packed with Fe₂(BDP)₃, followed by the monobranched isomers and finally linear *n*-hexane. Configurational-bias Monte Carlo simulations confirm the origins of the molecular separation.

Metal-organic frameworks constitute a large family of microporous solids exhibiting high surface areas, tunable pore

dimensions, and adjustable surface functionality (1–3). Consequently, their performance is beginning to rival traditional solid adsorbents, such as

zeolites and activated carbons, for some key gas-storage (4–7) and molecular-separation (8–12) applications. With regard to the latter, the possibility of creating pore characteristics that cannot readily be achieved in zeolites or carbons expands the opportunities for molecular recognition. In the presence of the right surface environment, separations currently carried out inefficiently could potentially be performed with a substantially reduced energy cost (13). The efficient separation of alkane isomers by adsorption is especially challenging because the molecules are chemically inert and have similar polarizabilities (14), leaving shape as the main handle available for their differentiation. This separation is critical to

¹Department of Chemistry, University of California, Berkeley, CA 94720, USA. ²Van't Hoff Institute for Molecular Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, Netherlands. ³Center for Neutron Research, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA. ⁴Institute of Physics, University of Silesia, ul. Uniwersytecka 4, 40-007 Katowice, Poland. ⁵Chemical and Biomolecular Engineering, University of Delaware, Newark, DE 19716, USA. ⁶Dipartimento di Scienza e Alta Tecnologia, Università dell'Insubria, via Valleggio 11, I-22100 Como, Italy.

*Corresponding author. E-mail: r.krishna@uva.nl (R.K.); jrlong@berkeley.edu (J.R.L.)

the production of gasoline, which is composed of ~10% pentanes and hexanes (15). Herein, we report a metal-organic framework featuring sharply angled pore walls of a type not encountered in zeolites and capable of fractionating alkane isomers according to the degree of branching.

Hexanes of formula C_6H_{14} are generated at enormous scale through a catalytic isomerization reaction that results in a thermodynamically controlled product stream composed of 10 to 30% of each of the five different isomers (15). The value of a particular isomer as a component in the gasoline pool is related to its research octane number (RON) and is highest for the dibranched hexanes 2,3-dimethylbutane and 2,2-dimethylbutane, which have values of 105 and 94, respectively. The RONs for the monobranched isomers 2-methylpentane and 3-methylpentane are substantially lower, at 74 and 75, respectively, whereas the value for linear *n*-hexane is only 30. To achieve higher octane number blends, current processes sieve *n*-hexane by using zeolites, generating a mixture of the other four isomers with a final RON of nearly 83 while returning *n*-hexane to the isomerization reactor (15–17). Additionally, some separation processes achieve higher-grade mixtures by subsequently distilling the monobranched isomers away from the valuable dimethylbutane products. Currently, about two million barrels of pentanes and hexanes are processed daily (15).

An improved hexane-separation process would selectively isolate the most valuable products, 2,3-dimethylbutane and 2,2-dimethylbutane, while returning the less valuable monobranched isomers to the isomerization reactor along with *n*-hexane (Fig. 1) (18). Further, it would potentially benefit public health, because it could reduce the usage of toxic aromatics, which are currently added to boost the octane number of gasoline (19). Performing this separation at or near the isomerization temperature would save a great deal of energy in the production of high-quality gasoline. Numerous attempts have been made to identify solid adsorbents capable of effecting an efficient separation of hexane isomers according to octane number, including zeolites (20, 21), silicas (22), and, very recently, metal-organic frameworks

(18, 23, 24). Although zeolite BETA facilitates the separation of 2,2-dimethylbutane from the monobranched isomers and some 2,3-dimethylbutane (the highest value isomer) elutes with it, these results are only seen at 0.01 bar and are therefore not useful for a separation (21). The many studies that have examined the adsorption or separation of some hexane isomers generally do not include the crucially important separation of both 2,2-dimethylbutane and 2,3-dimethylbutane from the monobranched and linear isomers.

In comparing the structures of zeolites to those possible in metal-organic frameworks, a prominent distinction lies in the angles that can be obtained for the internal pore walls. Whereas the pore contours defined by a zeolite scaffolding are necessarily obtuse, owing to the O-Si-O and Si-O-Si angles of ~109° and >130°, respectively (25), the higher coordination numbers possible for the metal nodes within a metal-organic framework can give rise to flat pore surfaces that intersect at acute angles. An extreme example of this is apparent in the structure of the new metal-organic framework $Fe_2(BDP)_3$ (Fig. 2), which we obtained as a black microcrystalline solid upon reaction of $Fe(acetylacetonate)_3$ and 1,4-benzenedipyrazole (H_2BDP) in *N,N*-dimethylformamide (26). Here, octahedral iron(III) centers are linked via μ^2 -pyrazolate units to form chains running along one crystal axis (Fig. 2C), where both nitrogens on one ring bind to different iron atoms. The rigid, nearly planar BDP^{2-} ligands (Fig. 2B) define a channel structure featuring sharply angled crevices running along the triangle corners. The chains of octahedral iron(III) centers form the vertices of these triangles. One can readily envision how these crevices might provide strong van der Waals contacts for linear alkanes, whereas branched alkanes would protrude into the pores. The framework of $Fe_2(BDP)_3$ is directly analogous to the carboxylate-linked structure of $Sc_2(BDC)_3$ (BDC^{2-} = 1,4-benzenedicarboxylate) (27) but with a larger metal-metal triangle-edge length of 13.25(2) Å as a result of the longer BDP^{2-} linker. The structure is also related to that of $Co(BDP)$, wherein chains of tetrahedral cobalt(II) centers lead instead to square channels (28).

The strong iron(III)-pyrazolate bonds and highly connected architecture of $Fe_2(BDP)_3$ lend it exceptional chemical and thermal stability. The material can be boiled in aqueous solutions at pH = 2 to 10 for 2 weeks (fig. S1) or heated in air to at least 280°C (fig. S2) without losing crystallinity. Heating the solvated, as-synthesized form of $Fe_2(BDP)_3$ at 180°C under dynamic vacuum afforded a completely activated, microporous form of the compound exhibiting a Brunauer-Emmett-Teller surface area of 1230 m²/g.

Pure-component equilibrium adsorption isotherms for the five different hexane isomers were measured for $Fe_2(BDP)_3$ at temperatures of 130°, 160°, and 200°C (Fig. 3), within the range of 100° to 200°C relevant to the industrial separation (15). In contrast to zeolite 5A, which operates as a sieve for separating *n*-hexane, the dimensions of the channels in the evacuated structure of $Fe_2(BDP)_3$ are large enough to accommodate all five hexane isomers. At 130°C, the isotherm data rise with varying degrees of steepness until reaching saturation. The decreasing degrees of steepness in the isotherms for linear versus monobranched versus dibranched isomers indicate a corresponding decrease in the adsorption strength. At 200°C, the isotherms rise considerably less steeply and do not reach saturation. At 160°C, *n*-hexane and the monobranched isomers exhibit the behavior expected of an interpolation between the 130° and 200°C isotherms: Saturation capacity is reached or nearly reached, but at pressures higher than those required at 130°C. At about 100 mbar and 200°C, $Fe_2(BDP)_3$ adsorbs 60% more *n*-hexane by volume and 100% more by weight than zeolite 5A (29). This enhancement, together with the adsorption selectivity discussed further below, renders $Fe_2(BDP)_3$ a more efficient adsorbent than zeolite 5A for the *n*-hexane separation presently carried out in industry.

At 160°C, the two dibranched hexane isomers display stepwise adsorption with an inflection point near 100 mbar. The stepwise uptake of alkanes has been observed previously with cyclohexane (30) and *n*-hexane (31) adsorption, and can be explained with entropic arguments, as supported by calorimetric data (32). Here, the inflection point occurs near 0.5 moles of guest per mole of $Fe_2(BDP)_3$. As supported by the simulations discussed below, rearrangement occurs at this loading based on packing around the structural ridge that is created along the triangular channel by two adjacent $Fe_2(BDP)_3$ subunits (Fig. 2D). This behavior is not evident at 200°C because a loading of 0.5 mole is not attained under the conditions measured. The step is not as prominent at 130°C for two reasons: (i) The entropic component of the Gibbs free energy is smaller at lower temperatures, and (ii) the steepness of the isotherm obscures the feature.

Complementary to the ordered adsorption of dibranched isomers, neutron diffraction data demonstrate the lack of a preferred arrangement of *n*-hexane molecules within the pores of $Fe_2(BDP)_3$. Diffraction patterns were collected at 10 K on microcrystalline powders loaded with 0.5 and 1.0

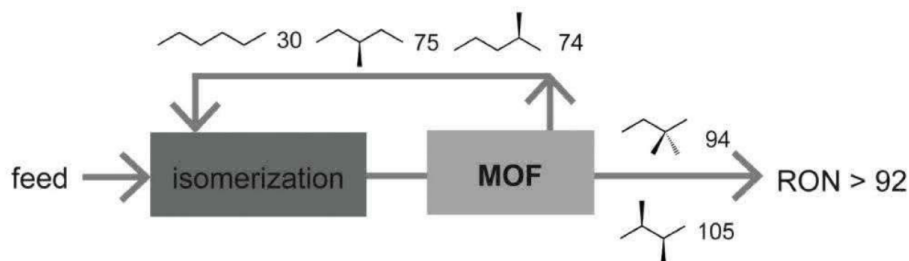


Fig. 1. Illustration of the proposed future hexane isomer separation processes. The individual RON of each isomer and the average RON of the final product are included. Current technology uses the small cross-sectional area of *n*-hexane and a zeolite-sieving process to remove this low-RON isomer from the mixture and return it to the isomerization reactor. In some cases, distillation is then used to further augment the RON of the final product. Given the adsorption selectivity that can be attained within a metal-organic framework (MOF) as demonstrated here for $Fe_2(BDP)_3$, it may now be viable to separate only the two most valuable, dibranched isomers from the other three.

molar equivalents of perdeuterated *n*-hexane. Attempts to refine the position of the *n*-hexane molecules in both samples were thoroughly exhausted

by using several different refinement approaches, suggesting that there is unlikely any correlation between the positions of guests adsorbed in each pore.

In addition to entropic differences, the enthalpies of adsorption among the five hexane isomers in $\text{Fe}_2(\text{BDP})_3$ are dependent on the degree

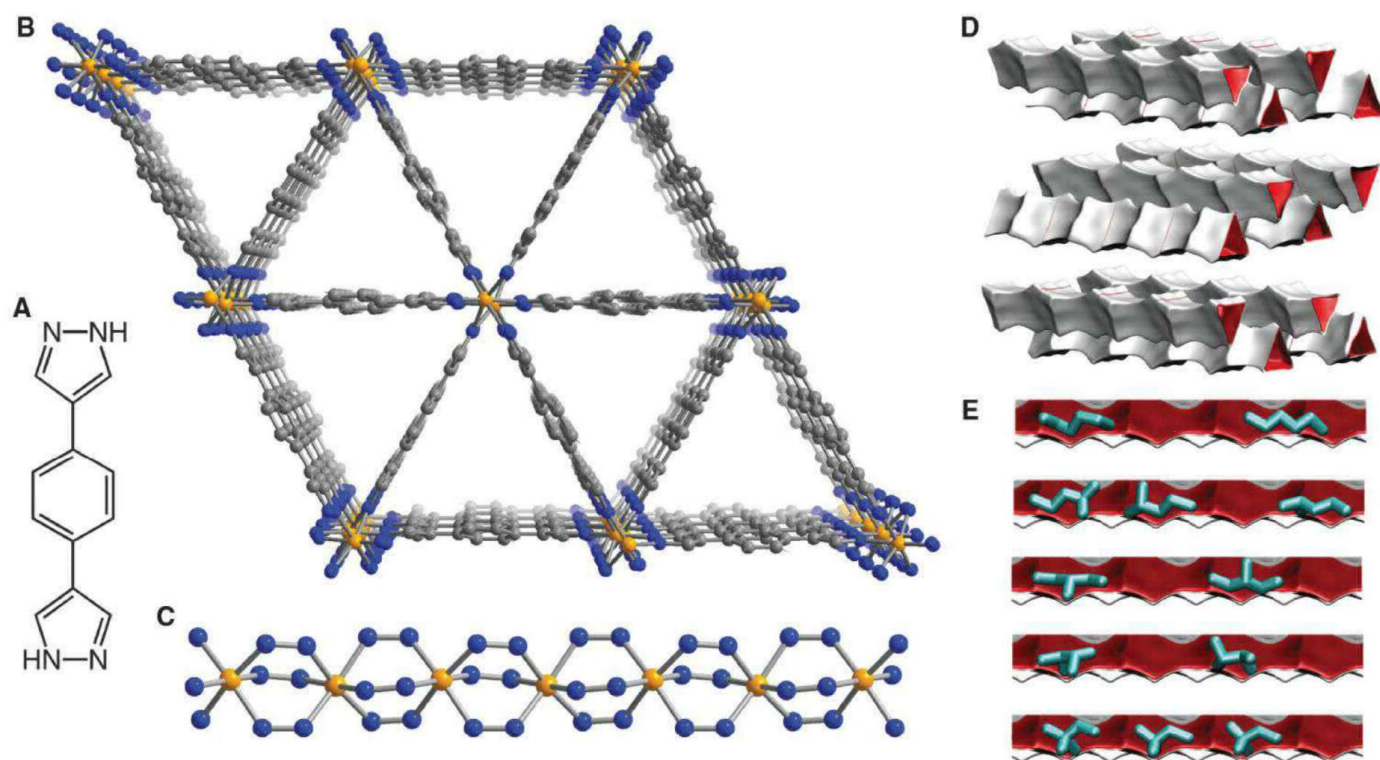
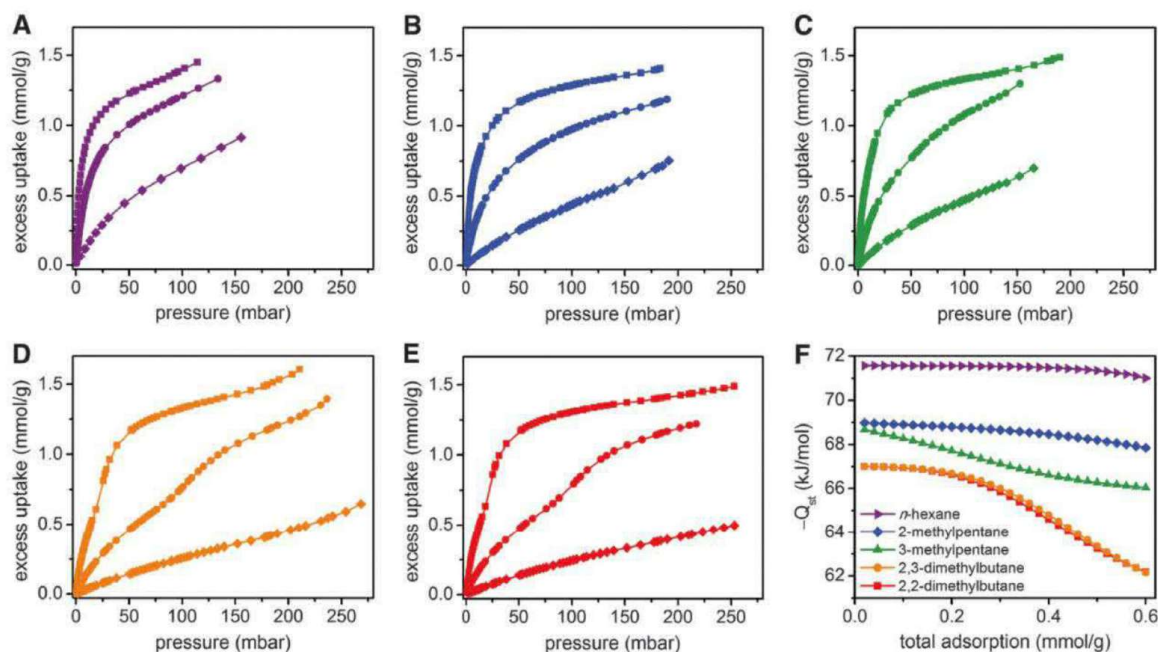


Fig. 2. Chemical structures. Depiction of the bridging ligand precursor H_2BDP (A) and portions of the structure of $\text{Fe}_2(\text{BDP})_3$ as determined by analysis of powder x-ray diffraction data. Orange, blue, and gray spheres represent Fe, N, and C atoms, respectively; H atoms are omitted for clarity. (B) The view along the [001] direction in the orthorhombic crystal structure (space group $Fddd$). (C) A perpendicular view of the one-dimensional chains of pyrazolate-bridged Fe^{III} octahedra, excluding C and H atoms. Selected interatomic distances and angles are as follows: for Fe-N1x, 1.98 Å; Fe-N2, 2.04 Å;

Fe-N1, 1.94 Å; Fe...Fe (vertex to vertex), 13.25 Å; Fe-Fe (nearest neighbor), 3.85 Å; N1x-Fe-N2, 7°; N1x-Fe-N2', 92.5°; N1x-Fe-N1, 81.2°; N1x-Fe-N1', 88.9°; N1-Fe-N2, 91.6°; Fe-N1x-N1x, 123.1°; Fe-N2-N1, 115.6°; Fe-N1-N2, 131.0°. Typical estimated standard deviations are 0.01 Å for bond distances and 0.1° for bond angles. (D) The van der Waals surfaces associated with the corrugated triangular channels running through the structure. (E) Snapshots of the hexane isomers within the channels of $\text{Fe}_2(\text{BDP})_3$ for a loading of four molecules per unit cell at 160°C, as observed in CBMC simulations.

Fig. 3. Pure-component equilibrium adsorption isotherms.

Gas adsorption isotherms for (A) *n*-hexane, (B) 2-methylpentane, (C) 3-methylpentane, (D) 2,3-dimethylbutane, and (E) 2,2-dimethylbutane in $\text{Fe}_2(\text{BDP})_3$ at 130°C (squares), 160°C (circles), and 200°C (diamonds). Lines represent dual-site Langmuir-Freundlich fits to these data (table S2). Isothermic heats of adsorption (Q_{st}) calculated from these isotherm data are plotted in (F) as a function of loading.



of branching as well. Isothermic heats of adsorption (Fig. 3F) were calculated by differentiation of the temperature-independent, combined dual-site Langmuir-Freundlich fits to the isotherm data obtained at 130°, 160°, and 200°C. In order to report enthalpy values obtained without extrapolation, the results are only plotted up to the highest loading attained at the highest temperature. The linear *n*-hexane isomer has the strongest interaction with the framework, because a greater fraction of its surface can interact with the triangular channel pore surface than the other isomers. Comparing methylpentane isomers, the zero-coverage isosteric heat is initially very similar, but as more guests enter the pores, the strength of interaction of the two isomers diverges. Here, the higher flexibility of the 2-methylpentane chain apparently allows a stronger adsorbate-adsorbent interaction to be maintained at higher loadings. The more compact dimethylbutane isomers are not flexible enough to maximize van der Waals interactions with the pore surfaces and have the lowest enthalpies at all loadings. This trend in selectivity has occasionally been observed at very low loadings in zeolites as a result of different isomers residing in different parts of a heterogeneous pore structure (33), but at more substantial loadings it disappears. In contrast, for $\text{Fe}_2(\text{BDP})_3$, the isomers are all interacting with the same, relatively homogeneous surface differently. This trend has also been observed in other metal-organic frameworks for a selected subset of hexane isomers (23, 34) but never for all five isomers.

Taken together, the trends in the enthalpy and entropy of adsorption for the five hexane isomers conspire to generate a free energy hierarchy of linear < monobranched < dibranched isomer adsorption. The linear isomers bind more strongly and additionally do not require substantial reorganization at loadings above 0.5 molecules per unit cell. Although separate enthalpy (35) and entropy (36) trends of this type have been established previously, the combined contributions of both within $\text{Fe}_2(\text{BDP})_3$ offers exceptional potential for separating the valuable dibranched hexanes from the other isomers.

The hexane isomer separation ability of $\text{Fe}_2(\text{BDP})_3$ was evaluated with a breakthrough experiment in which an equimolar mixture of all five isomers in N_2 was passed over a bed of the material heated at 160°C. As shown in Fig. 4A, pure 2,2-dimethylbutane eluted from the bed, followed by 2,3-dimethylbutane. These dibranched isomers are the most desirable, owing to their high RON values. Monobranched 2-methylpentane eluted subsequently, immediately followed by 3-methylpentane and then, much later, linear *n*-hexane. The RON of the product mixture leaving the column is also plotted in Fig. 4A, simplified as a weighted average of the RONs of each component (16). During the beginning of the breakthrough experiment, the RON of the eluted mixture rises to greater than 90, significantly higher than the value of 83 that is typical for industrially refined hexane blends (15).

The shape of the breakthrough curve for each isomer is informative with regard to the separation (18). The steepness of the dimethylbutane breakthrough events suggests that the separations for these isomers result from essentially equilibrium processes and are not diffusion controlled. As shown in Fig. 3F, the enthalpy of adsorption for the two dimethylbutane isomers is essentially identical up to a loading of 0.6 mmol/g. If diffusion is not the primary cause of the separation and the material is saturated at the breakthrough event, the strength of adsorption of these isomers presumably diverges at higher loadings, with 2,3-dimethylbutane adsorbing more strongly. The methylpentanes and *n*-hexane display more gradual breakthrough events, suggesting that diffusion is a contributory factor in their elution dynamics.

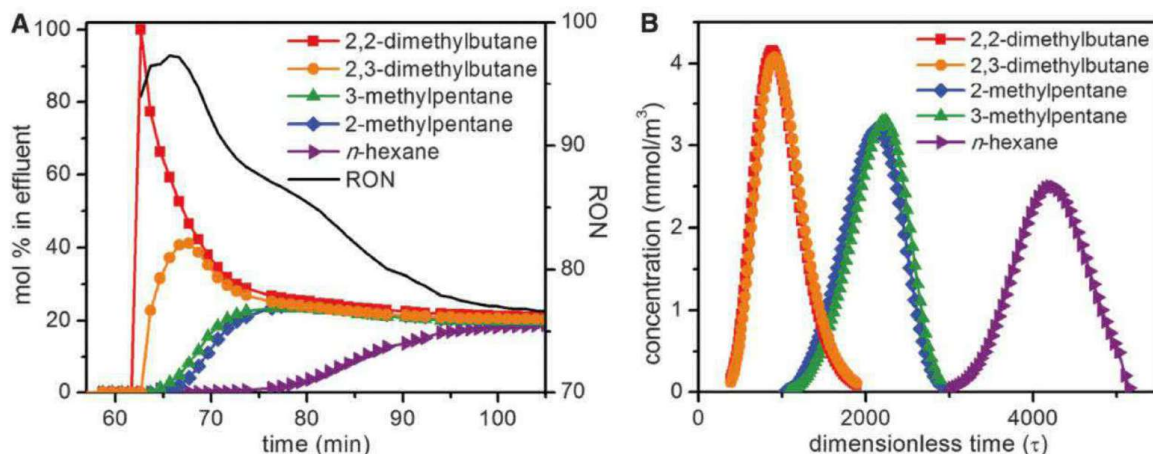
Pulse chromatography simulations (26) were performed to further probe the separation properties of $\text{Fe}_2(\text{BDP})_3$. The results (Fig. 4B) indicate that the material can separate a mixture of hexane isomers into three fractions, consisting of the two dimethylbutane isomers, the two methylpentane isomers, and, finally, pure *n*-hexane. These three fractions could potentially serve independent purposes: The dibranched isomers would be added

to gasoline, the *n*-hexane would be reintroduced to the isomerization reactor as it is presently, and the monobranched isomers could be returned to the isomerization reactor at a downstream location, allowing for better conversion (fig. S3). It is expected that such a staged recycling scheme could significantly boost the efficiency of the isomerization process.

Configurational-bias Monte Carlo (CBMC) simulations illustrate and support the mechanism of hexane isomer fractionation in $\text{Fe}_2(\text{BDP})_3$. The methodology used has been described in detail (37), and the Lennard-Jones parameters used for the atoms of the framework are specified in table S1. The results are presented in Fig. 2E as snapshots of the locations and conformations of hexane isomers adsorbed within the triangular channels of the structure. The snapshots are obtained from arbitrary channel segments, and so, although the simulated loading of four molecules per unit cell is the same overall for each isomer, the number of molecules in each channel differs among the images. The regularity of the 2,3-dimethylbutane and 2,2-dimethylbutane positions supports the hypothesis that these molecules have a preferred arrangement, one that requires energy input to overcome. The snapshots also validate the enthalpic argument that van der Waals overlap decreases with the degree of branching, a hypothesis previously put forth to explain the trends in the diffusion rates for pentane isomers in zeolite NaY (38). Generally, the hexane backbones align along the vertices of the triangular channels, which provide the maximum surface area for dispersion interactions. Figs. S4 and S5 include more arbitrary channel segments to provide more support for these arguments. From the observed conformations, it is evident that the number of carbon atoms that can effectively interact with the pore wall decreases with the degree of branching. The dimethylbutane isomers are more compact and have the weakest van der Waals interactions with the framework surface.

Comparisons with published data and further CBMC simulations demonstrate the unique efficacy of $\text{Fe}_2(\text{BDP})_3$ in separating hexane isomers

Fig. 4. Separation of an equimolar mixture of 2,2-dimethylbutane (red), 2,3-dimethylbutane (orange), 3-methylpentane (green), 2-methylpentane (blue), and *n*-hexane (purple) running through a packed bed of $\text{Fe}_2(\text{BDP})_3$ at 160°C. (A) Experimental breakthrough data, together with the RON calculated for the eluted mixture at 1 bar. (B) The pulsed chromatogram calculated on the basis of the isotherm data presented in Fig. 3.



according to the degree of branching. A detailed comparison of its performance relative to zeolites and other metal-organic frameworks is provided in section 2.4 of the supplementary materials. A realistic comparison, of relevance to industrial operations, is obtained by comparing the number of moles of 92 RON product that can be obtained per liter of adsorbent in a packed bed adsorber, taking account of diffusional limitations. These comparisons show that for $\text{Fe}_2(\text{BDP})_3$ the 92 RON productivity is 0.54 mol/l, whereas the values obtained for other adsorbents are consistently lower.

Very recently, a series of carboxylate-linked metal-organic frameworks with triangular channels was reported (39). Although these could potentially provide surrogates or even more efficient replacements for $\text{Fe}_2(\text{BDP})_3$, our CMBC calculations suggest that the size of the channels in $\text{Fe}_2(\text{BDP})_3$ is nearly optimal for a hexane isomer separation, as described in detail in section 2.4.14 of the supplementary materials. Narrower triangular channels cannot accommodate all five isomers, whereas wider channels do not maximize the differences in van der Waals contacts.

CBMC simulations indicate that $\text{Fe}_2(\text{BDP})_3$ is similarly suitable for separating pentane and heptane isomers according to the degree of branching. For pentane isomers, the results indicate the hierarchy of adsorption strengths as n -pentane > 2-methylbutane > neopentane (fig. S6A); for heptanes, they follow the order n -heptane > 2-methylhexane $\approx$ 3-methylhexane > 2,2-dimethylpentane $\approx$ 2,3-dimethylpentane (fig. S6D). Accordingly, pulse chromatography simulations reveal the extraordinary ability of $\text{Fe}_2(\text{BDP})_3$ to separate the di-branched alkanes with high RON values from a mixture of pentane, hexane, and heptane isomers (fig. S7).

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Supplementary Materials

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Galvanic Replacement Reactions in Metal Oxide Nanocrystals

Myoung Hwan Oh,^{1,2*} Taekyung Yu,^{3*} Seung-Ho Yu,^{1,2} Byungkwon Lim,⁴ Kyung-Tae Ko,⁵ Marc-Georg Willinger,⁶ Dong-Hwa Seo,⁷ Byung Hyo Kim,^{1,2} Min Gee Cho,^{1,2} Jae-Hoon Park,^{5,8} Kisuk Kang,⁷ Yung-Eun Sung,^{1,2} Nicola Pinna,^{2,9,10†} Taeghwan Hyeon^{1,2†}

Galvanic replacement reactions provide a simple and versatile route for producing hollow nanostructures with controllable pore structures and compositions. However, these reactions have previously been limited to the chemical transformation of metallic nanostructures. We demonstrated galvanic replacement reactions in metal oxide nanocrystals as well. When manganese oxide (Mn_3O_4) nanocrystals were reacted with iron(II) perchlorate, hollow box-shaped nanocrystals of $\text{Mn}_3\text{O}_4/\gamma\text{-Fe}_2\text{O}_3$ ("nanoboxes") were produced. These nanoboxes ultimately transformed into hollow cage-like nanocrystals of $\gamma\text{-Fe}_2\text{O}_3$ ("nanocages"). Because of their nonequilibrium compositions and hollow structures, these nanoboxes and nanocages exhibited good performance as anode materials for lithium ion batteries. The generality of this approach was demonstrated with other metal pairs, including $\text{Co}_3\text{O}_4/\text{SnO}_2$ and $\text{Mn}_3\text{O}_4/\text{SnO}_2$.

The galvanic replacement reaction is the most versatile method of preparing hollow metallic nanostructures with controllable pore structures and compositions (1–7). These

reactions involve a corrosion process that is driven by the difference in the electrochemical potentials of two metallic species. The hollow interior is generated from the oxidative dissolution of

the metal nanocrystals (NCs) that are used as reactive templates. This strategy has also been used for the production of hollow semiconductor nanostructures (8). However, the chemical transformation of ionic systems via galvanic reactions has remained elusive. We demonstrated that a galvanic replacement reaction can occur in oxide NCs as well and can produce hollow oxide nanostructures.

Hollow oxide NCs have attracted much interest because of their potential for application in energy storage, catalysis, and medicine (9, 10). Considerable advances have been made in the synthesis of hollow oxide and semiconductor NCs (11–13). The Kirkendall effect has been exploited to produce complex hollow nanostructures of metal oxides and chalcogenides (11, 14–16). However, synthesizing hollow NCs of multivalent oxides still remains a substantial challenge (17, 18). We showed that by using a nanoscale galvanic replacement reaction, monometallic oxide NCs could be completely transformed into hollow multimetallic oxide nanostructures. Contrary to what occurs in metallic systems, a redox-couple reaction between multivalent metallic ions

took place, replacing the higher-oxidation-state ions in the NCs with lower-oxidation-state metal ions from solution. We focus here on one example, hollow heterostructured NCs of manganese oxide/iron oxide ($\text{Mn}_3\text{O}_4/\gamma\text{-Fe}_2\text{O}_3$) with a box-like shape ("nanoboxes"), which we transformed into cage-shaped iron oxide ($\gamma\text{-Fe}_2\text{O}_3$) NCs ("nanocages"). We also showed that these hollow-structured multimetallic oxide nanostructures exhibit synergistic properties that make them attractive for use as anode materials in lithium ion batteries (LIBs).

Mn_3O_4 NCs were prepared by slightly modifying a previously reported method (19, 20). A transmission electron microscope (TEM) image of the as-prepared Mn_3O_4 NCs showed that they were square prism-shaped, with sides of ~ 20 nm and a height of ~ 10 nm (Fig. 1A and fig. S1) (20). A high-resolution TEM (HRTEM) image and the corresponding Fourier transform (FT) pattern of the Mn_3O_4 NCs revealed that their top and side surfaces were enclosed by $\{001\}$ and $\{100\}$ facets, respectively, and their corners and edges were slightly truncated (Fig. 1B).

The galvanic replacement reaction was initiated by adding an aqueous iron(II) perchlorate solution into a suspension of the Mn_3O_4 NCs in xylene and subsequently heating the reaction mixture at 90°C for 1.5 hours (20). The reaction seemed to take place at the organic/aqueous interface of reverse vesicles (fig. S2) (20). Figure 1, C and D, shows the TEM and HRTEM images of the product derived from the galvanic replacement reaction between the Mn_3O_4 NCs and 1 ml of 2.0 M iron(II) perchlorate solution. The original NCs transformed completely into $\gamma\text{-Fe}_2\text{O}_3$ nanocages with a hollow interior and holes on their shell. The exterior shape of the nanocages was nearly the same as that of the original Mn_3O_4 NCs. The average particle size increased from 19 to 23 nm after the galvanic reaction, because of the formation of the $\gamma\text{-Fe}_2\text{O}_3$ shell (fig. S3) (20). The shell of these nanocages had a single-

crystalline structure with highly ordered continuous lattice fringes (Fig. 1D). Inductively coupled plasma-atomic emission spectrometry (ICP-AES) showed that the molar ratio of Fe to Mn in the $\gamma\text{-Fe}_2\text{O}_3$ nanocages was 91:9 (Fig. 1E) and that almost complete metal replacement took place. We could also synthesize hollow nanocages of $\text{Co}_3\text{O}_4/\text{SnO}_2$ and $\text{Mn}_3\text{O}_4/\text{SnO}_2$ from the reactions of Co_3O_4 and Mn_3O_4 NCs with aqueous tin(II) chloride solutions (fig. S4) (20), demonstrating the generality of the transformation process. These nanocages exhibited polycrystalline structures, because the crystal structure of SnO_2

(rutile) is different from that of Co_3O_4 and Mn_3O_4 (spinel).

Surface precipitation of $\gamma\text{-Fe}_2\text{O}_3$ reduces the dissolution rate of Mn_3O_4 by preventing the diffusion of ions and electrons to its surface (21). However, if the volume of Mn_3O_4 was below a certain critical limit, its complete dissolution and thus the near-total replacement of Mn by Fe could be achieved. Indeed, the ICP-AES results showed that the Mn molar ratio decreased to less than 0.1% when the Mn_3O_4 NCs were treated with more concentrated 3.0 M iron(II) perchlorate. The volume of Mn_3O_4 affected this transformation; for

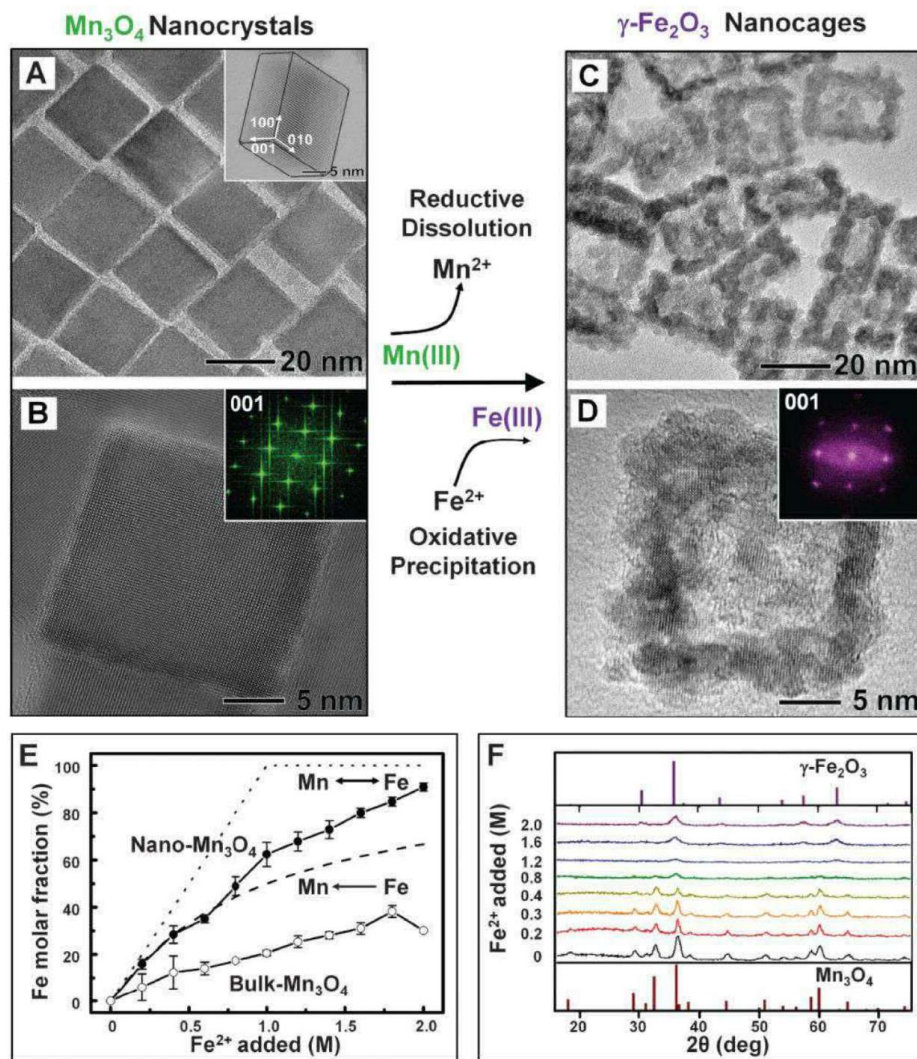


Fig. 1. (A and B) TEM image of Mn_3O_4 NCs. (A) Low-magnification image of Mn_3O_4 NCs. The inset shows the corresponding HRTEM image of a single NC recorded along the $[111]$ axis. (B) HRTEM image of a single Mn_3O_4 NC recorded along the $[001]$ axis. The inset shows the corresponding FT pattern. (C and D) (C) Low-magnification TEM and (D) HRTEM image of the $\gamma\text{-Fe}_2\text{O}_3$ nanocages synthesized by reacting the Mn_3O_4 NCs with 1 ml of 2.0 M aqueous iron(II) perchlorate solution. The inset shows the corresponding FT pattern. (E) ICP-AES data showing the molar fraction of Fe in the reaction product as a function of the amount of iron(II) perchlorate added during the synthesis (solid circles) and their bulk counterpart (open circles). The molar fractions expected in the cases of the complete replacement of Mn by Fe (dotted line) and the addition of Fe to Mn (dashed line) are also shown. (F) Powder XRD patterns of the original Mn_3O_4 NCs and for the samples synthesized by the reaction with Mn_3O_4 NCs with 1 ml of aqueous solutions of iron(II) perchlorate having different concentrations. For comparison, the standard XRD patterns of Mn_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$ are also shown.

¹Center for Nanoparticle Research, Institute for Basic Science (IBS), and School of Chemical and Biological Engineering, Seoul National University, Seoul 151-742, Korea. ²World Class University (WCU) Program of Chemical Convergence for Energy and Environment and School of Chemical and Biological Engineering, Seoul National University, Seoul 151-742, Korea. ³Department of Chemical Engineering, College of Engineering, Kyung Hee University, Yongin 446-701, Korea. ⁴School of Advanced Materials Science and Engineering, Sungkyunkwan University, Suwon 440-746, Korea. ⁵Cross-Coupled Complex Materials Research and Department of Physics, Pohang University of Science and Technology, Pohang 790-784, Korea. ⁶Department of Inorganic Chemistry, Fritz Haber Institute of the Max Planck Society, Berlin 14195, Germany. ⁷Department of Materials Science and Engineering, Research Institute of Advanced Materials, Seoul National University, Seoul 151-742, Korea. ⁸Division of Advanced Materials Science, Pohang University of Science and Technology, Pohang 790-784, Korea. ⁹Department of Chemistry and Centre for Research in Ceramics & Composite Materials, University of Aveiro, Aveiro 3810-193, Portugal. ¹⁰Institut für Chemie, Humboldt-Universität zu Berlin, Berlin 12489, Germany.

*These authors contributed equally to this work.

†Corresponding author. E-mail: thyeon@snu.ac.kr (T.H.); nicola.pinna@hu-berlin.de (N.P.)

the same reaction performed with bulk Mn_3O_4 powder, the molar fraction of Fe in the thus-formed product was substantially lower (less than 25%) (Fig. 1E). Powder x-ray diffraction (XRD) patterns of the reactant and the products (Fig. 1F) confirmed that the tetragonally distorted Mn_3O_4 spinel transformed into a cubic $\gamma\text{-Fe}_2\text{O}_3$ spinel as the concentration of the iron(II) perchlorate solution was increased. Superconducting quantum interface device measurements showed that magnetization of the NCs increased steadily with an increase in the Fe content (fig. S5) (20).

The evolution of the internal hollow core was examined by reacting the Mn_3O_4 NCs with aqueous iron(II) perchlorate solutions of various concentrations (Fig. 2 and fig. S6) (20). When a less concentrated solution was used, the cores of the Mn_3O_4 NCs were dissolved partially, and nanoboxes with relatively thick walls were formed (Fig. 2, B, C, F, and G). The images in Fig. 2, B and F, and fig. S7 (20) show that pinholes were formed on the surface of the nanoboxes. These observations indicate that pores develop inside the NCs by a mechanism analogous to pinhole corrosion, in which pinholes serve as transport paths during the dissolution of the NC core. As

the concentration of iron(II) perchlorate was increased to 1.0 M, the pores increased further in size, and the nanoboxes evolved into nanocages with clear openings (Fig. 2H). During the intermediate stages of the replacement reaction, both the nanoboxes and the nanocages exhibited continuous fringe patterns. Figure 2, B to E, shows a slight increase in the interplanar distance between (100) of Mn_3O_4 NCs and (110) of $\gamma\text{-Fe}_2\text{O}_3$ NCs. This change, however, did not alter the alignments of the lattices along the square basal planes of the NCs, demonstrating topotactic transformation. The color-mapped energy-filtered TEM (EFTEM) images in the inset of Fig. 2G and fig. S8 (20) show the accumulation of Fe species in the shell of the nanoboxes, whereas the image in Fig. 2I shows the deposition of Fe species over the entire surface of the nanocages. We did not observe any noticeable amount of Mn species in the $\gamma\text{-Fe}_2\text{O}_3$ shell of the nanoboxes (Fig. 2C). These results suggest that the Kirkendall effect does not play a major role in the current hollowing process (figs. S9 and S10), probably because of the low reaction temperature of 90°C , where the interdiffusion rates of Mn and Fe ions are low (table S1) (20).

The galvanic reaction observed in this study could be driven by the difference in the standard reduction potentials of $\text{Fe}^{3+}/\text{Fe}^{2+}$ (0.77 V) and $\text{Mn}_3\text{O}_4/\text{Mn}^{2+}$ (1.82 V) pairs, in which Fe^{2+} ions reduce Mn^{3+} ions in the Mn_3O_4 NCs (fig. S11) (20). Indeed, the Mn_3O_4 NCs were not transformed into hollow nanocages when reacted with cobalt(II) perchlorate (fig. S12) (20), which can be attributed to the higher standard reduction potential of the $\text{Co}^{3+}/\text{Co}^{2+}$ pair (1.87 V) than that of the $\text{Mn}_3\text{O}_4/\text{Mn}^{2+}$ pair.

The mechanism of redox replacement was collaborated by measurement of the valence state with x-ray absorption spectroscopy (XAS) and x-ray magnetic circular dichroism (XMCD) measurements (fig. S13) (20). The Mn $L_{2,3}$ -edge x-ray absorption spectra of the nanoboxes were almost identical to those of the original Mn_3O_4 NCs. The Mn^{3+} peaks gradually disappeared with increasing Fe^{2+} concentration ($[\text{Fe}^{2+}]$), and only Mn^{2+} peaks remained after the complete replacement reaction (22). However, the decrease in $[\text{Mn}^{2+}]$ to less than 9% suggested that most of the Mn^{2+} were also removed from the tetrahedral sites. Both XAS and XMCD spectra at the Fe $L_{2,3}$ -edges of the nanocages were similar to those of $\gamma\text{-Fe}_2\text{O}_3$

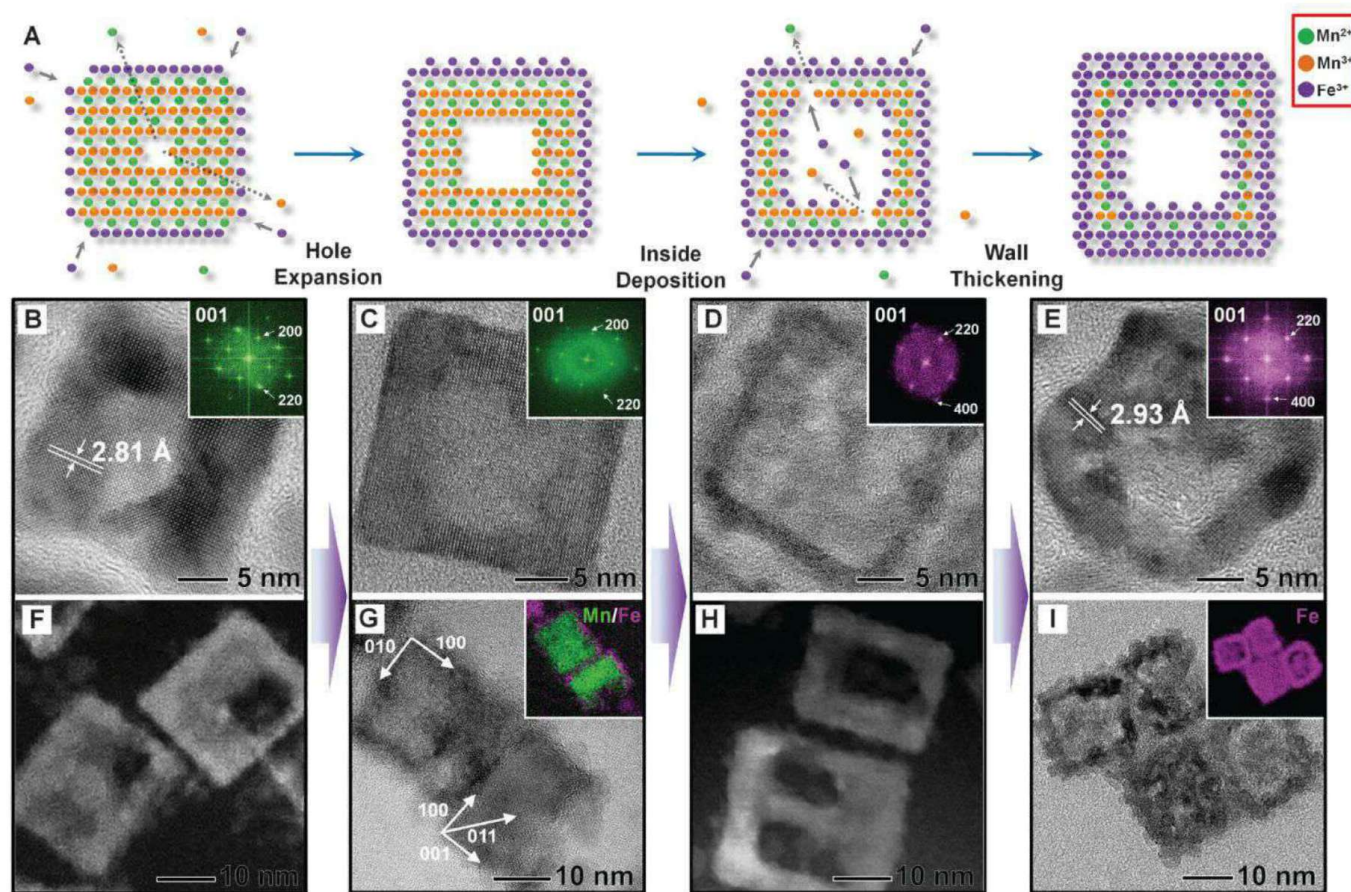


Fig. 2. (A) Schematic illustration of the transformation of Mn_3O_4 NCs, showing the evolution of their morphology via the localized dissolution of Mn_3O_4 and the surface precipitation of $\gamma\text{-Fe}_2\text{O}_3$. **(B to E)** HRTEM images of the hollow nanostructures synthesized by the reaction of Mn_3O_4 NCs with 1 ml of aqueous solutions of iron(II) perchlorate having different concentrations: **(B)** 0.4 M,

(C) 0.6 M, **(D)** 1.0 M, and **(E)** 1.6 M. Insets show the corresponding FT patterns. **(F)** High-angle annular dark-field scanning TEM (HAADF-STEM) image of the nanoboxes shown in **(B)**. **(G)** TEM image and a corresponding EFTEM image of the nanoboxes shown in **(C)**. **(H)** HAADF-STEM image of the nanocages shown in **(D)**. **(I)** TEM image and a corresponding EFTEM image of the nanocages shown in **(E)**.

that contained only Fe^{3+} , but not to those of Fe_3O_4 that contained both Fe^{3+} and Fe^{2+} .

When the galvanic reaction is initiated, some of Mn_3O_4 at the surface is dissolved into the solution (fig. S14) (20). The precipitation of $\gamma\text{-Fe}_2\text{O}_3$ started with the outermost shell of the Mn_3O_4 NCs, preventing the outward Mn^{2+} diffusion. This transformation occurred preferentially around the edges of the shell (Fig. 2G, inset) (23). An increase in the number of empty octahedral sites caused by dissolution of the reduced Mn^{2+} could lead to the complete disruption of the remaining lattice that consists of tetrahedral Mn^{2+} and oxygen anions (24). This process formed pinholes during the initial stage of reaction in the areas not covered by the $\gamma\text{-Fe}_2\text{O}_3$ layer; i.e., in the middle of the basal plane. As the reaction progresses, the electrons released from the Fe^{2+} migrate inward and reduce the octahedral Mn^{3+} in the interior. The pinholes formed facilitated the dissolution of the residual core species. Because of the open structure of the nascent $\gamma\text{-Fe}_2\text{O}_3$ nanocages, precipitation of $\gamma\text{-Fe}_2\text{O}_3$ occurred in their hollow interior at later stages. We also observed the formation of isolated $\gamma\text{-Fe}_2\text{O}_3$ NCs during the galvanic reaction (fig. S8A) (20), indicating that some of oxidized Fe^{3+} ions are converted to $\gamma\text{-Fe}_2\text{O}_3$ NCs via homogeneous nucleation. Together with

the initial dissolution of Mn_3O_4 at the surface, the formation of isolated $\gamma\text{-Fe}_2\text{O}_3$ NCs may account for the generation of relatively thin shells of $\gamma\text{-Fe}_2\text{O}_3$, although the overall amounts of the dissolved Mn species and the precipitated Fe species are similar (fig. S15) (20). The perchlorate moiety in the aqueous iron(II) perchlorate solution did not contribute to the formation of the hollow structure, because no morphological change was observed when the Mn_3O_4 NCs were reacted with perchloric acid (fig. S16) (20). In addition, we found that cagelike structures with a different morphology could also be produced via the same method by using different-shaped Mn_3O_4 NCs as the starting material (fig. S17) (20).

Transition-metal oxides such as Mn_3O_4 and Fe_3O_4 are attractive anode materials for LIBs because of their higher specific capacities as compared to typical graphitic anodes. However, their capacities decrease drastically with cycling because of their severe volume changes during Li insertion and extraction (25, 26). Despite its theoretically high capacity (936 mAh g^{-1}), Mn_3O_4 usually exhibits a much lower capacity ($\leq \sim 400 \text{ mAh g}^{-1}$) because of its low electrical conductivity ($\sim 10^{-7}$ to $10^{-8} \text{ S cm}^{-1}$) (25). The current galvanic replacement reactions in oxide NCs can be a promising technique for engineering their

structures and tuning their chemical compositions to improve their electrochemical properties for their applications to LIB anodes. Our hollow nanoboxes and nanocages were coated with polypyrrole in situ in the reaction solution and carbonized at 500°C for 2 hours in an Ar atmosphere (20). Through the thermal treatment, the samples transformed into solid solutions of Mn_3O_4 and Fe_3O_4 (i.e., $\text{Mn}_{3-x}\text{Fe}_x\text{O}_4$) with a ferrite structure and varying compositions ranging from $\text{Mn}_{2.0}\text{Fe}_{1.0}\text{O}_4$ to $\text{Mn}_{0.3}\text{Fe}_{2.7}\text{O}_4$ (Fig. 3A), while retaining their original hollow structures (Fig. 3B and fig. S18) (20).

For $\text{Mn}_{2.0}\text{Fe}_{1.0}\text{O}_4$, two plateaus were observed around 0.55 and 0.68 V in the first discharge curve, with a peak ratio of 2:1, as shown in the inset of Fig. 3C. $\text{Mn}_{1.5}\text{Fe}_{1.5}\text{O}_4$ and $\text{Mn}_{1.1}\text{Fe}_{1.9}\text{O}_4$ exhibited two plateaus at slightly higher voltages of around 0.59 and 0.73 V, respectively. The ratio of the plateau at the upper potential gradually increased with the Fe content of the sample. For $\text{Mn}_{0.6}\text{Fe}_{2.4}\text{O}_4$, the plateau around 0.60 V eventually disappeared and the upper-potential plateau shifted to 0.83 V, a value similar to that of commercial Fe_3O_4 ($< 50 \text{ nm}$, no. 637106 from Aldrich) (Fig. 3C), indicating that almost all Mn ions were replaced by Fe ions. The changes in the ratios and potentials of the two plateaus seem to be related

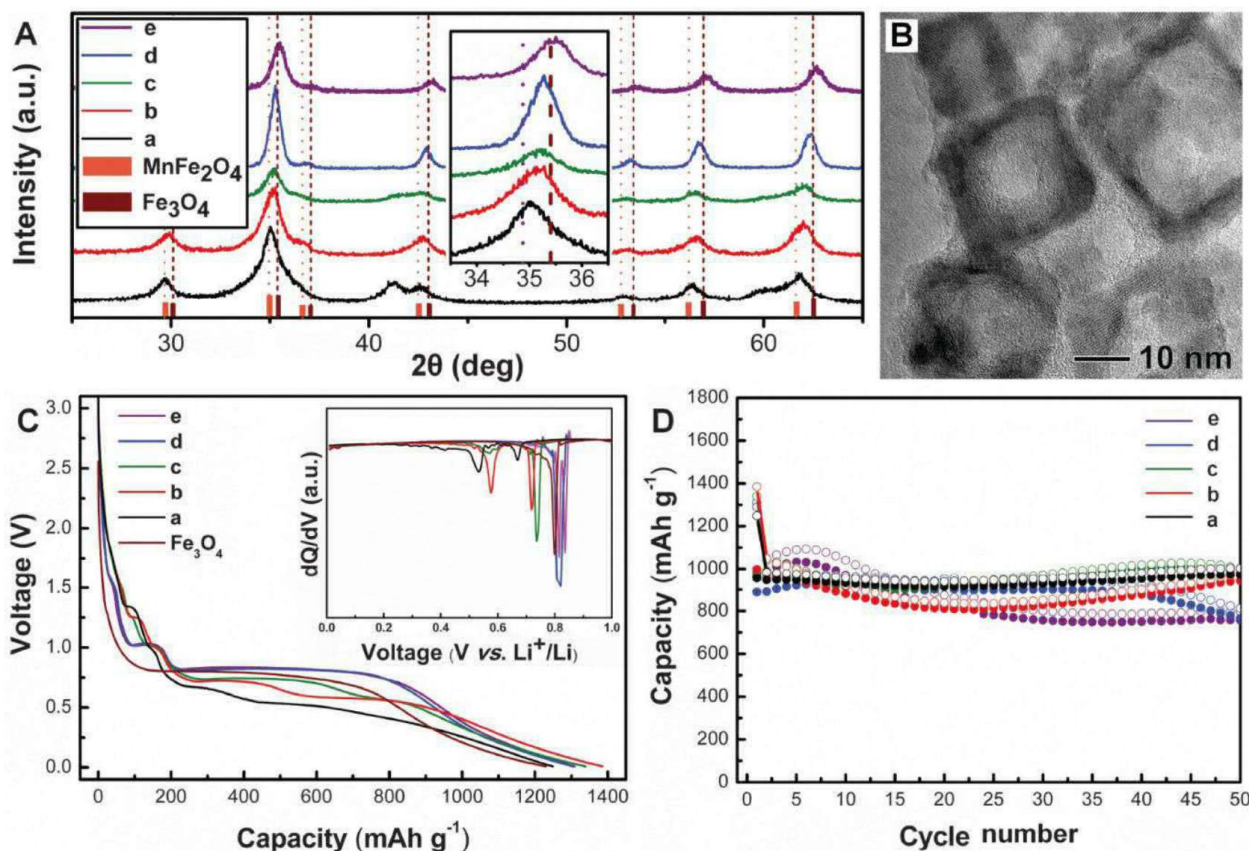


Fig. 3. Characterization and LIB anode applications of carbon-coated hollow $\text{Mn}_{3-x}\text{Fe}_x\text{O}_4$ NCs: (a) $\text{Mn}_{2.0}\text{Fe}_{1.0}\text{O}_4$, (b) $\text{Mn}_{1.5}\text{Fe}_{1.5}\text{O}_4$, (c) $\text{Mn}_{1.1}\text{Fe}_{1.9}\text{O}_4$, (d) $\text{Mn}_{0.6}\text{Fe}_{2.4}\text{O}_4$, and (e) $\text{Mn}_{0.3}\text{Fe}_{2.7}\text{O}_4$. (A) XRD patterns of the samples. a.u., arbitrary units. (B) TEM image of the carbon-coated $\text{Mn}_{1.1}\text{Fe}_{1.9}\text{O}_4$ NCs. (C and D) Electrochemical

measurements of the samples: (C) first discharge curves and corresponding results of the dQ/dV analysis in the inset, and (D) cycle performances at a current density of 100 mA g^{-1} in the voltage range from 3.0 to 0.01 V. Open symbols indicate discharge (lithiation) cycles, and solid symbols are charge (de-lithiation) cycles.

to the content of substituted Fe in Mn_3O_4 . The potential of $\text{Mn}_{3-x}\text{Fe}_x\text{O}_4$ as a function of the Fe content was investigated through density functional theory calculations, which indicated that a solid solution of $\text{Mn}_{3-x}\text{Fe}_x\text{O}_4$ can occur between Mn_3O_4 and Fe_3O_4 (fig. S20) and that their average reaction voltages are proportional to the amount of the Fe_3O_4 component from 0.400 to 0.818 V at $0 \leq x \leq 3$ (table S2) (20). Our results suggest that the potentials of multicomponent spinel electrode materials can be easily tuned by galvanic replacement reaction and subsequent thermal treatment.

Figure 3D shows cycle performances of hollow $\text{Mn}_{3-x}\text{Fe}_x\text{O}_4$ NCs. $\text{Mn}_{1.1}\text{Fe}_{1.9}\text{O}_4$ exhibited first discharge and charge capacities of 1339 and 984 mAh g^{-1} , respectively. $\text{Mn}_{1.1}\text{Fe}_{1.9}\text{O}_4$ exhibited a high reversible capacity of $\sim 1000 \text{ mAh g}^{-1}$ with almost no fading up to 50 cycles. The reversible capacities and cyclic stabilities of $\text{Mn}_{2.0}\text{Fe}_{1.0}\text{O}_4$ and $\text{Mn}_{1.5}\text{Fe}_{1.5}\text{O}_4$ were comparable to those of $\text{Mn}_{1.1}\text{Fe}_{1.9}\text{O}_4$. Most of the samples showed higher specific capacities and better cyclic stabilities than those of carbon-coated solid $\text{Mn}_{0.8}\text{Fe}_{2.2}\text{O}_4$ NCs (fig. S21) (20). These good cyclic stabilities can be attributed to their hollow structure, which provides extra free space for alleviating the structural strain caused by the large volume change (26, 27) and additional sites for lithium ion storage (28). $\text{Mn}_{0.6}\text{Fe}_{2.4}\text{O}_4$ and $\text{Mn}_{0.3}\text{Fe}_{2.7}\text{O}_4$, which are close to Fe_3O_4 , showed less cyclic stability than the other samples, indicating that multi-metallic composition contributes to the enhanced cyclic stabilities (29). Furthermore, $\text{Mn}_{1.1}\text{Fe}_{1.9}\text{O}_4$ exhibited the highest rate capability (fig. S22)

(20), which is due to the improvement in its electronic conductivity resulting from the mixed valency of the multicomponent $\text{Mn}_{3-x}\text{Fe}_x\text{O}_4$ (30).

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Supplementary Materials

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The Cells and Circuitry for Itch Responses in Mice

Santosh K. Mishra and Mark A. Hoon*

Itch is triggered by somatosensory neurons expressing the ion channel TRPV1 (transient receptor potential cation channel subfamily V member 1), but the mechanisms underlying this nociceptive response remain poorly understood. Here, we show that the neuropeptide natriuretic polypeptide b (Nppb) is expressed in a subset of TRPV1 neurons and found that $\text{Nppb}^{-/-}$ mice selectively lose almost all behavioral responses to itch-inducing agents. Nppb triggered potent scratching when injected intrathecally in wild-type and $\text{Nppb}^{-/-}$ mice, showing that this neuropeptide evokes itch when released from somatosensory neurons. Itch responses were blocked by toxin-mediated ablation of Nppb-receptor-expressing cells, but a second neuropeptide, gastrin-releasing peptide, still induced strong responses in the toxin-treated animals. Thus, our results define the primary pruriceptive neurons, characterize Nppb as an itch-selective neuropeptide, and reveal the next two stages of this dedicated neuronal pathway.

Pruritic responses are triggered by somatosensory neurons, with several itch-inducing agents acting through pathways involving the ion channel TRPV1 (transient receptor po-

tential cation channel subfamily V member 1) (1, 2) and the effector enzyme PLCβ3 (3). Recently, agonist-induced silencing of TRPV1-expressing neurons was shown to result in a profound loss of all itch responses (2) but also affects thermosensation and pain (4). In contrast, a much more specific loss of pruriception was observed in mice lacking the gastrin-releasing peptide (GRP) receptor (5) and in animals in which the spinal interneurons expressing this receptor had been

specifically targeted with a GRP-conjugated toxin (GRP-saporin) (6). Thus, it was hypothesized that GRP is the neurotransmitter that initiates a labeled line for itch (5, 6).

Previously, we generated mice that had lost all TRPV1-lineage neurons (7). These mice have major pruritic deficits (Fig. 1A) as well as a complete loss of thermosensory input (7), which is in keeping with previous reports that used capsaicin-induced lesions (2, 4). To identify candidate molecules that might mediate itch signaling, we used a differential microarray-based screen that identified many TRPV1-enriched transcripts (table S1). Among these, natriuretic polypeptide b (Nppb) is prominently expressed in a small subset of dorsal root ganglia (DRG) neurons but is dramatically decreased in sensory ganglia from TRPV1-DTA animals (Fig. 1B). Double-label in situ hybridization (ISH) explicitly demonstrates that all Nppb-expressing neurons contain TRPV1 (Fig. 1C) and PLCβ3 (Fig. 1D), which are critically required for histamine-induced scratching in mice (2, 3). Moreover, double labeling (Fig. 1, E and F) shows almost complete overlap between the expression of Nppb and two Mas-related G protein-coupled receptors that have recently been shown to detect specific pruritogens (8–10).

We generated $\text{Nppb}^{-/-}$ animals (Fig. 2A) and showed that these mutants displayed no detect-

Molecular Genetics Unit, Laboratory of Sensory Biology, National Institute of Dental and Craniofacial Research (NIDCR), NIH, Building 49, Room 1A16, 49 Convent Drive, Bethesda, MD 20892, USA.

*Corresponding author. E-mail: mark.hoon@nih.gov

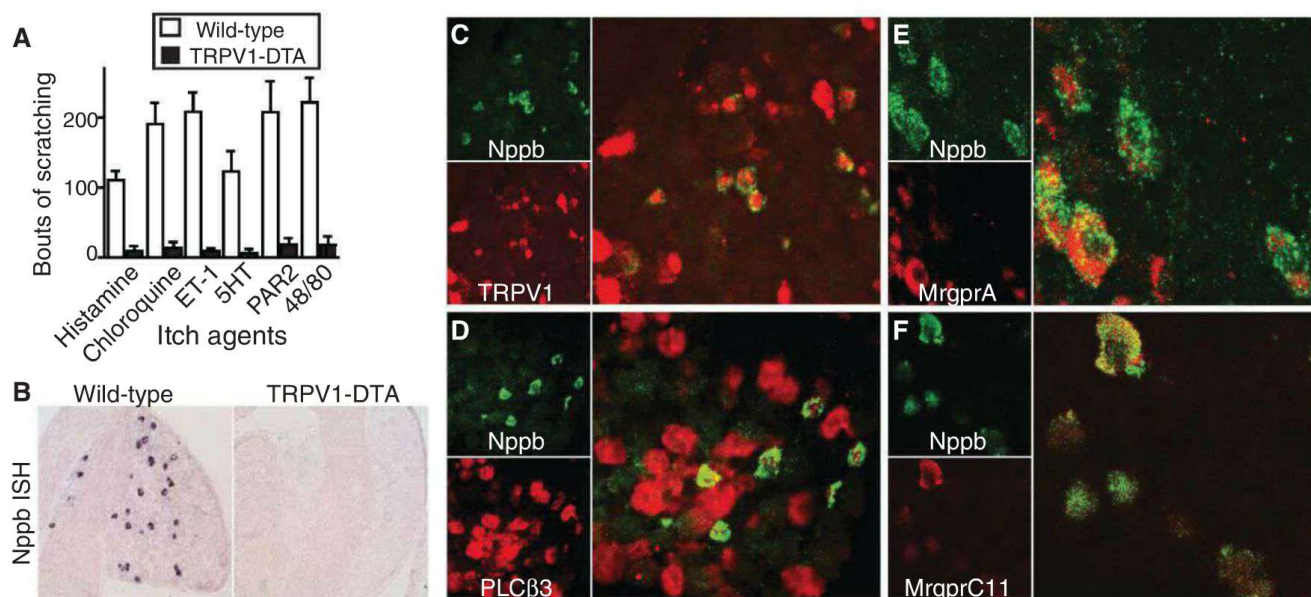


Fig. 1. Nppb is always coexpressed with itch-related signaling molecules TRPV1 and PLCβ3. (A) TRPV1-DTA mice exhibit dramatically reduced scratch responses after intradermal injection of pruritic agents; histamine (100 μ g in 10 μ l); chloroquine (100 μ g); endothelin 1 (1 μ M); methyl-serotonin (30 μ g); PAR2 agonist SLIGRL-NH₂ (100 μ g); and compound 48/80 (100 μ g). Itch-inducing substances were injected intradermally into the shoulder of mice, and numbers of scratching bouts were assessed over 30 min; data are mean $\pm$ SEM ($n \geq 7$ animals) normalized to wild-type litter controls. (B) ISH of sections through DRG illustrating loss of Nppb-expression in TRPV1-DTA animals. Quantitation of Nppb-expressing neurons revealed that $7 \pm 0.6\%$ of NeuN-positive C4 DRG neurons express the neuropeptide in wild-type mice

($n = 3$ animals). (C) Double-label ISH of DRG demonstrates that Nppb (green) and TRPV1 (red) are coexpressed in the same sensory neurons; only a subset of TRPV1-expressing neurons contain Nppb. (D) All Nppb-positive neurons also express PLCβ3 (red), but many PLCβ3 neurons are Nppb-negative. (E) Double-label ISH also shows that Nppb-positive neurons (green) all express MrgprA receptors (including MrgprA3, the receptor for chloroquine; red), with more than 70% of MrgprA-expressing neurons also containing the neuropeptide. (F) Double-label immunostaining demonstrates complete overlap between expression of MrgprC11 (red), the receptor for the pruritogen SLIGRL-NH₂, and Nppb (green) in somatosensory neurons. Additional characterization of neurons expressing Nppb is available in fig. S2.

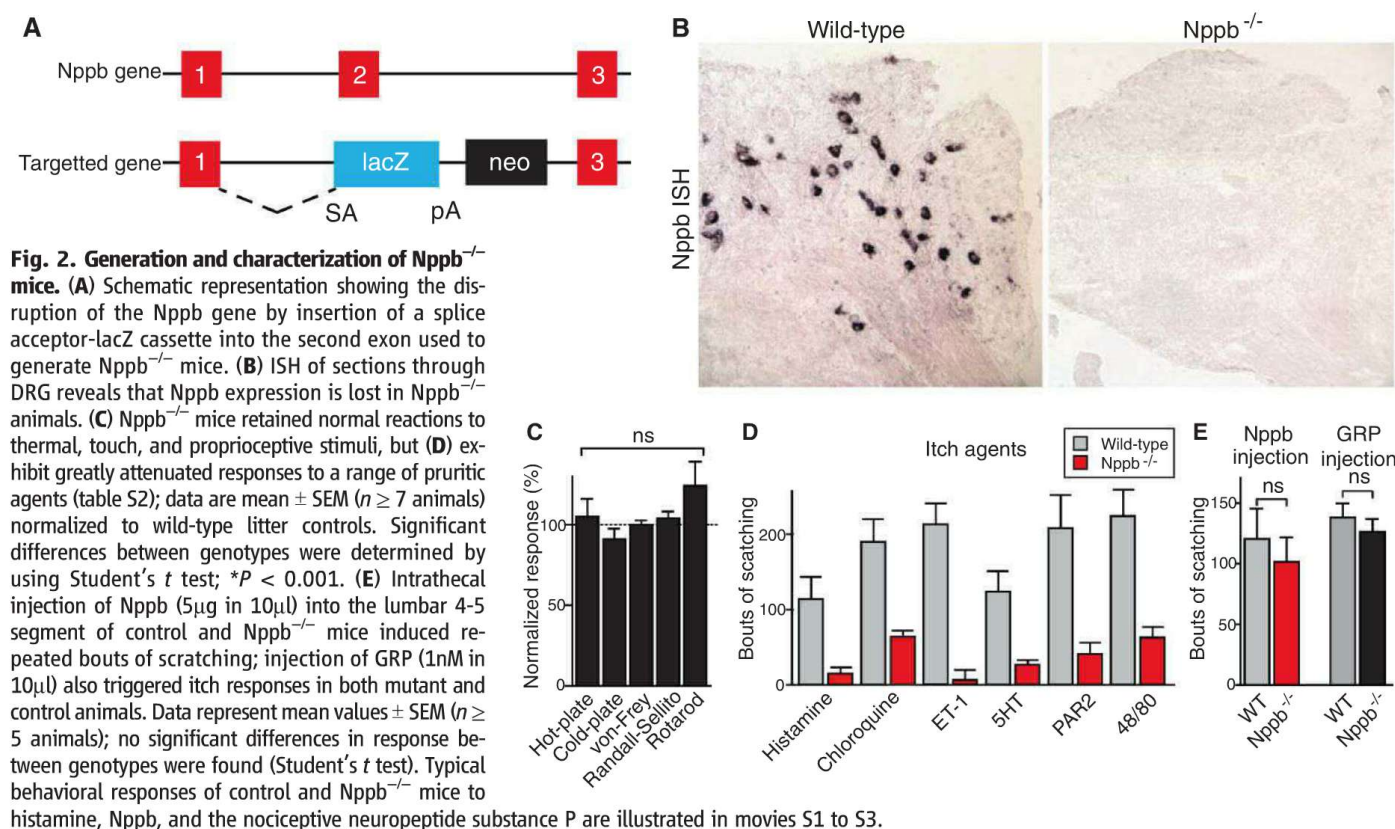


Fig. 2. Generation and characterization of Nppb^{-/-} mice. (A) Schematic representation showing the disruption of the Nppb gene by insertion of a splice acceptor-lacZ cassette into the second exon used to generate Nppb^{-/-} mice. (B) ISH of sections through DRG reveals that Nppb expression is lost in Nppb^{-/-} animals. (C) Nppb^{-/-} mice retained normal reactions to thermal, touch, and proprioceptive stimuli, but (D) exhibit greatly attenuated responses to a range of pruritic agents (table S2); data are mean $\pm$ SEM ($n \geq 7$ animals) normalized to wild-type litter controls. Significant differences between genotypes were determined by using Student's *t* test; $*P < 0.001$. (E) Intrathecal injection of Nppb (5 μ g in 10 μ l) into the lumbar 4-5 segment of control and Nppb^{-/-} mice induced repeated bouts of scratching; injection of GRP (1 nM in 10 μ l) also triggered itch responses in both mutant and control animals. Data represent mean values $\pm$ SEM ($n \geq 5$ animals); no significant differences in response between genotypes were found (Student's *t* test). Typical behavioral responses of control and Nppb^{-/-} mice to histamine, Nppb, and the nociceptive neuropeptide substance P are illustrated in movies S1 to S3.

able expression of Nppb (Fig. 2B). The mice were healthy, had normal numbers of nociceptive, touch, and proprioceptive neurons, and the distribution and number of dorsal horn interneurons was unaffected by gene disruption (fig. S1). Nppb^{-/-} mice retained normal responses to thermal, nociceptive, touch, and proprioceptive stimulation when tested with standard assays (Fig. 2C). We performed intradermal injections and recorded numbers of scratching bouts for substances that directly activate pruriceptors and with compound 48/80 that causes itch via an indirect route (11). All of these agents (table S2) reliably triggered multiple bouts of scratching in control animals (Fig. 2D, gray bars), but Nppb^{-/-} mice were almost completely insensitive to the full range of pruritic substances tested (Fig. 2D, red bars).

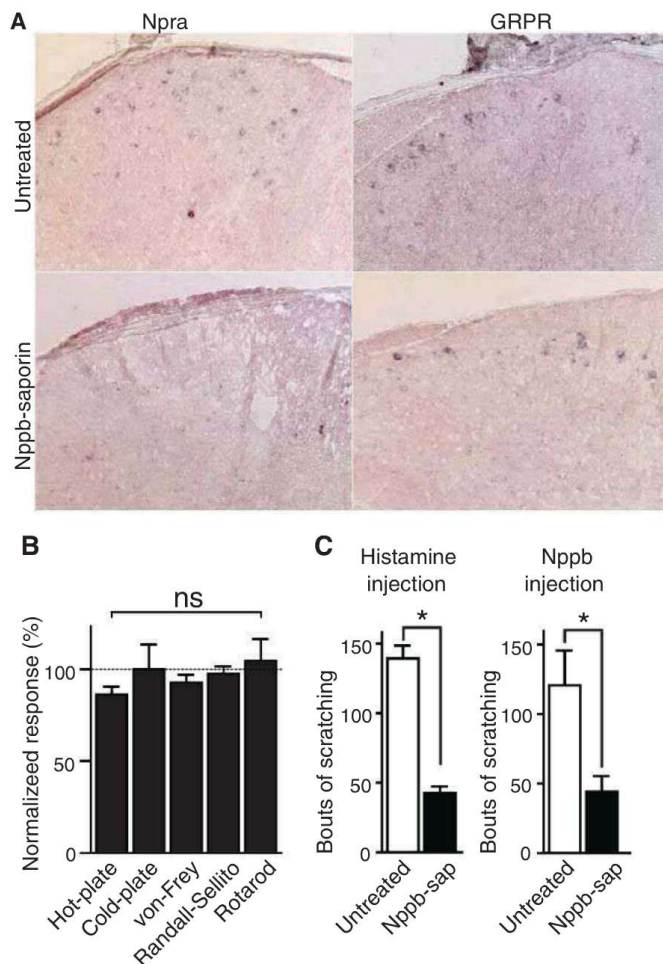
How does Nppb induce this stereotyped scratch response? We reasoned that because this peptide is prominently expressed in somatosensory neurons, the most plausible explanation for its role would be if it acted as a specific itch-related neuromodulator (or neurotransmitter) in the spinal cord. Indeed, intrathecal injection of Nppb induced profound scratching behavior in wild-type animals (Fig. 2E, gray bar), demonstrating that spinal-Nppb is sufficient to induce itch even without activation of the peripheral neurons that express it. Intrathecal injection of Nppb into Nppb^{-/-} mice also led to an equivalent phenotype (Fig. 2E, red bar). Loss of Nppb in sensory afferents was thus responsible for the pruriceptive deficits seen in mutant mice. Therefore, we suggest that Nppb-expression delineates the subset of somatosensory neurons that detect pruritic agents and that central release of Nppb from these neurons is necessary for the itch response.

Because Nppb is responsible for transmitting the peripheral signals that trigger pruritic responses, its receptor Npra (12) should be expressed at the site of afferent fiber synaptic connections in the spinal dorsal horn and mark the secondary neurons in the itch-response circuit. Therefore, we used ISH to localize Npra expression in the dorsal horn and found that it is indeed expressed in a limited subset of neurons (most likely interneurons), primarily in the outer layer—lamina I (Fig. 3A, left)—corresponding to the terminal field of TRPV1-expressing sensory neurons (13).

To examine whether the Npra neurons in the spinal cord function in the itch circuit and whether they are selectively required for pruritogen-induced scratching (rather than other somatosensory responses), we used a targeted-toxin cell-ablation strategy (14). We injected a Nppb-saporin conjugate intrathecally into wild-type mice so as to target their Npra-expressing cells and assessed the effectiveness, specificity, and behavioral consequences of administering this toxin. Approximately 70% of Npra-positive neurons were eliminated after administration of toxin (Fig. 3A and fig. S3). This targeting was highly selective, with neither cells expressing the GRP-receptor nor other dorsal horn interneurons affected by Nppb-saporin treat-

Fig. 3. Selective ablation of Npra receptor neurons in the spinal cord impairs pruriception.

(A) Expression of Npra in the dorsal spinal cord was assessed by using ISH. In normal mice (top), a subset of interneurons in the outer layer express Npra; however, after intrathecal administration of Nppb-saporin (bottom), few Npra neurons remained. In contrast, the number of GRP-receptor-positive cells (right) were unaffected by Nppb-saporin. (B) Nppb-saporin-treated mice retain normal reactions to thermal, touch, and proprioceptive stimuli, but (C) exhibit greatly attenuated responses to intradermal injection of histamine (100 μ g in 10 μ l) or intrathecal administration of Nppb. Data represent means normalized against untreated controls $\pm$ SEM ($n \geq 5$ animals). Significant differences between genotypes were determined by using Student's *t* test; **P* < 0.01.



ment (Fig. 3A and fig. S3). Toxin-injected mice displayed normal responses to thermal, touch, and painful stimulation (Fig. 3B). However, we observed a dramatic reduction in scratching evoked by histamine (Fig. 3C), indicating that these neurons are required for itch responses but not for other somatosensory pathways.

How do these findings fit with current models of itch behavior? The GRP-receptor has been shown to be a key element in the pruritic pathway (5, 6), with the suggestion that GRP might be the primary neurotransmitter for itch. However, this view has also been questioned (11, 15). We were unable to detect more than trace quantities of GRP expression in the DRG using a sensitive quantitative polymerase chain reaction (PCR) assay (Fig. 4A). Similarly, somatosensory neurons from GRP-reporter mice—Tg(GRP-EGFP)—were negative for enhanced green fluorescent protein (EGFP) expression (fig. S4A). In contrast, ISH for GRP and analysis of EGFP expression in the GRP-reporter mice revealed that this neuropeptide is present in a population of neurons in the dorsal horn (Fig. 4B and fig. S4A), as reported previously (15, 16). Therefore, we concluded that GRP cannot act at the level of pruriception but must function downstream of Nppb and so applied three complementary functional strategies

to substantiate this hypothesis and dissect the itch-response circuit.

First, we demonstrated that GRP-induced scratching was unaltered either by knocking out Nppb (Fig. 2E) or by the ablation of Npra-expressing neurons (Fig. 4D). Second, we showed that pharmacological inhibition of the GRP-receptor not only attenuated behavioral responses to the pruritic agent histamine or GRP injection (fig. S4B) but also inhibited scratching after intrathecal administration of Nppb (Fig. 4E). Last, we tested mice with selective ablation of GRP-receptor-expressing neurons and again found significantly reduced itch responses to Nppb (Fig. 4E). These data place GRP downstream of Nppb in the itch response circuit and suggest that the secondary pruriceptors are targets for one neuropeptide, Nppb, and in turn signal through a second peptide, GRP. Just as this model predicts, all Npra-expressing neurons in the dorsal horn contain GRP (Fig. 4F), and Nppb-saporin treatment significantly reduces the number of GRP-expressing cells (Fig. 4B, right).

Our results molecularly characterize the first three stations of an itch response pathway in mice (Fig. 4G), demonstrate that Nppb marks the primary sensory neurons, and show that this peptide is both necessary and sufficient for transmission

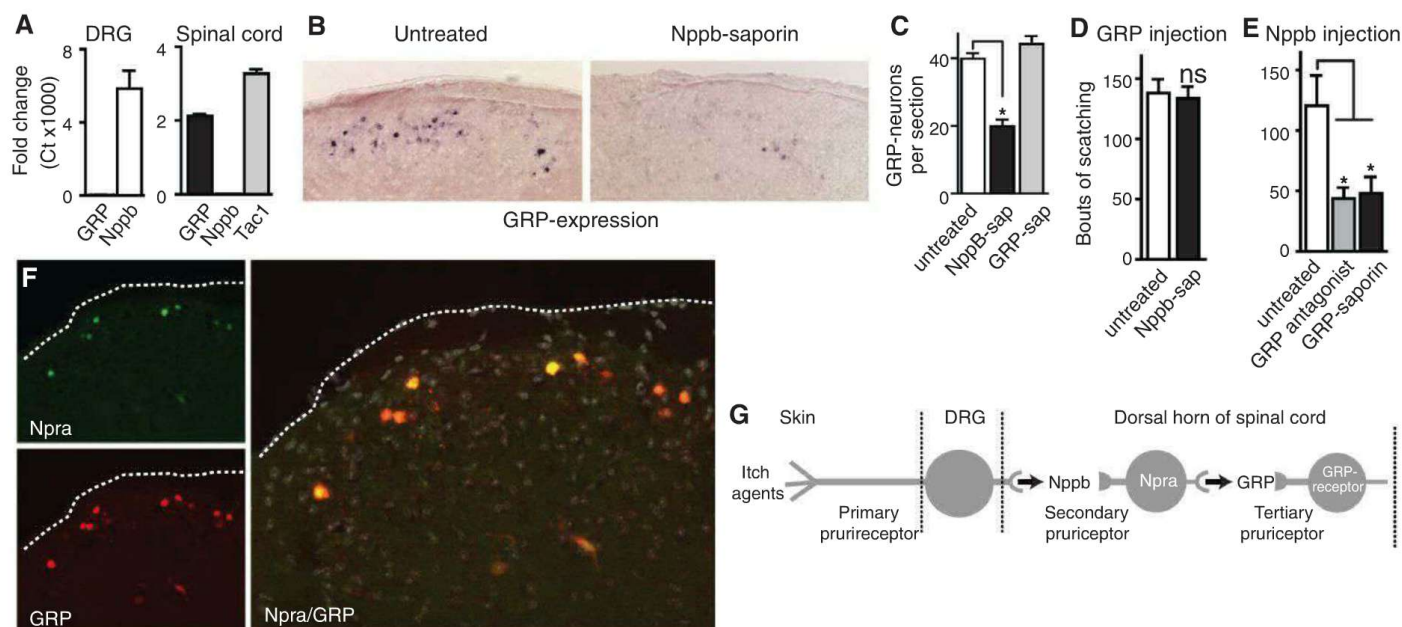


Fig. 4. GRP acts directly downstream of Nppb in the rodent pruriceptive circuit. (A) Quantitative PCR was used to quantitate expression of GRP and Nppb relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the DRG and spinal cord. GRP is robustly expressed in the spinal cord (at a level comparable with Tac1) but is almost undetectable in the DRG. Nppb is prominently expressed in DRG but is not present in the spinal cord. Data represent mean $\pm$ SEM for triplicate cDNA preparations each analyzed in two separate PCR reactions. (B) ISH was used to analyze GRP expression in the dorsal horn of normal (left) and Nppb-saporin treated mice (right); many GRP-expressing interneurons are lost on ablation of Npra-expressing cells. (C) A significant number of GRP neurons was eliminated after Nppb-saporin (Nppb-sap) treatment. GRP-saporin (GRP-sap), which targets GRP-receptor neurons, has no effect on the number of GRP interneurons; data represent mean $\pm$ SEM

($n \geq 4$ animals). Significant differences between groups were determined by using Student's *t* test; * $P < 0.001$. (D) Ablation of Npra neurons had no effect on intrathecally administered GRP-induced scratch responses. (E) Scratching induced by means of lumbar injection of Nppb was strongly attenuated by pretreatment with a selective GRP antagonist or by the ablation of GRP-expressing neurons with GRP-saporin. Data in (D) and (E) are mean $\pm$ SEM ($n \geq 6$ animals); * $P < 0.001$ (Student's *t* test). (F) Double-label immunohistochemistry was used to localize interneurons expressing Npra (green) and GRP-driven EGFP (red) in sections through the dorsal horn of Tg(EGFP) mice. (Left) Typical individual staining patterns. (Right) Merged Npra and GRP images, with the cell nuclei counterstained with DAPI (gray); the outline of the dorsal horn is dotted. (G) Model of the first three stages of the pruriceptive circuit, with the critical neuropeptide used at each stage indicated.

of peripheral signals that induce stereotypic itch responses. Unlike previously characterized receptors (8, 9) and signaling molecules (1–3) that affect the detection of particular itch-inducing agents, Nppb is necessary for responses to a wide range of pruritogens (compounds classed as inducing histamine- and nonhistamine-related itch) (table S2). Our data also refine the role GRP and GRP-receptor cells play in the itch-response pathway by placing them at later stages than had been hypothesized previously (5, 6). Many questions about itch remain unanswered, including its close relationship to sensing pain (17) and its slow kinetics and long duration, as well as reports from human and nonhuman primate studies that different central pathways mediate histamine and nonhistamine itch (18–21). Because Nppb is critically required for pruriception in mice, future studies involving ablation and/or activation of the Nppb-expressing somatosensory neurons together with circuit tracing may reveal whether these cells directly trigger a spinal scratch reflex, are selective detectors for the sensation of itch, or are in fact more broadly tuned nociceptors. Such experiments will help reveal the central mechanism (or mechanisms) for itch, explain the interactions

between pruriception and other somatosensory signals, and ultimately provide a powerful stimulus for the rational design of novel therapies to alleviate chronic itch.

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Supplementary Materials

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Changes in Taste Neurons Support the Emergence of an Adaptive Behavior in Cockroaches

Ayako Wada-Katsumata, Jules Silverman, Coby Schal*

In response to the anthropogenic assault of toxic baits, populations of the German cockroach have rapidly evolved an adaptive behavioral aversion to glucose (a phagostimulant component of baits). We hypothesized that changes in the peripheral gustatory system are responsible for glucose aversion. In both wild-type and glucose-averse (GA) cockroaches, D-fructose and D-glucose stimulated sugar-gustatory receptor neurons (GRNs), whereas the deterrent caffeine stimulated bitter-GRNs. In contrast, in GA cockroaches, D-glucose also stimulated bitter-GRNs and suppressed the responses of sugar-GRNs. Thus, D-glucose is processed as both a phagostimulant and deterrent in GA cockroaches, and this newly acquired peripheral taste sensitivity underlies glucose aversion in multiple GA populations. The rapid emergence of this highly adaptive behavior underscores the plasticity of the sensory system to adapt to rapid environmental change.

Sensory systems guide the assessment of food, habitat, and potential mates, and prominently govern intra- and interspecific inter-

actions. Although great progress has been made in our understanding of chemosensory processing, especially in insects (1, 2), how chemosensory

systems change in response to rapidly changing environments remains largely unknown. Cross-species divergence has been well investigated, particularly in olfactory processes (2–4). However, identifying the chemosensory mechanisms that underlie adaptive intraspecific polymorphisms has been challenging. Among the most important such polymorphisms are sensory adaptations that confer behavioral resistance to insecticides (5).

The German cockroach, *Blattella germanica*, offers a tractable system to explore mechanisms of sensory adaptation. Since the mid-1980s, control of this pest has increasingly shifted to baits that combine an insecticide with various phagostimulants, typically D-glucose (glucose henceforth) and D-fructose (fructose) (6). Within just several years, cockroach populations evolved a

Department of Entomology and W.M. Keck Center for Behavioral Biology, North Carolina State University, Campus Box 7613, Raleigh, NC 27695–7613, USA.

*Corresponding author. E-mail: coby@ncsu.edu

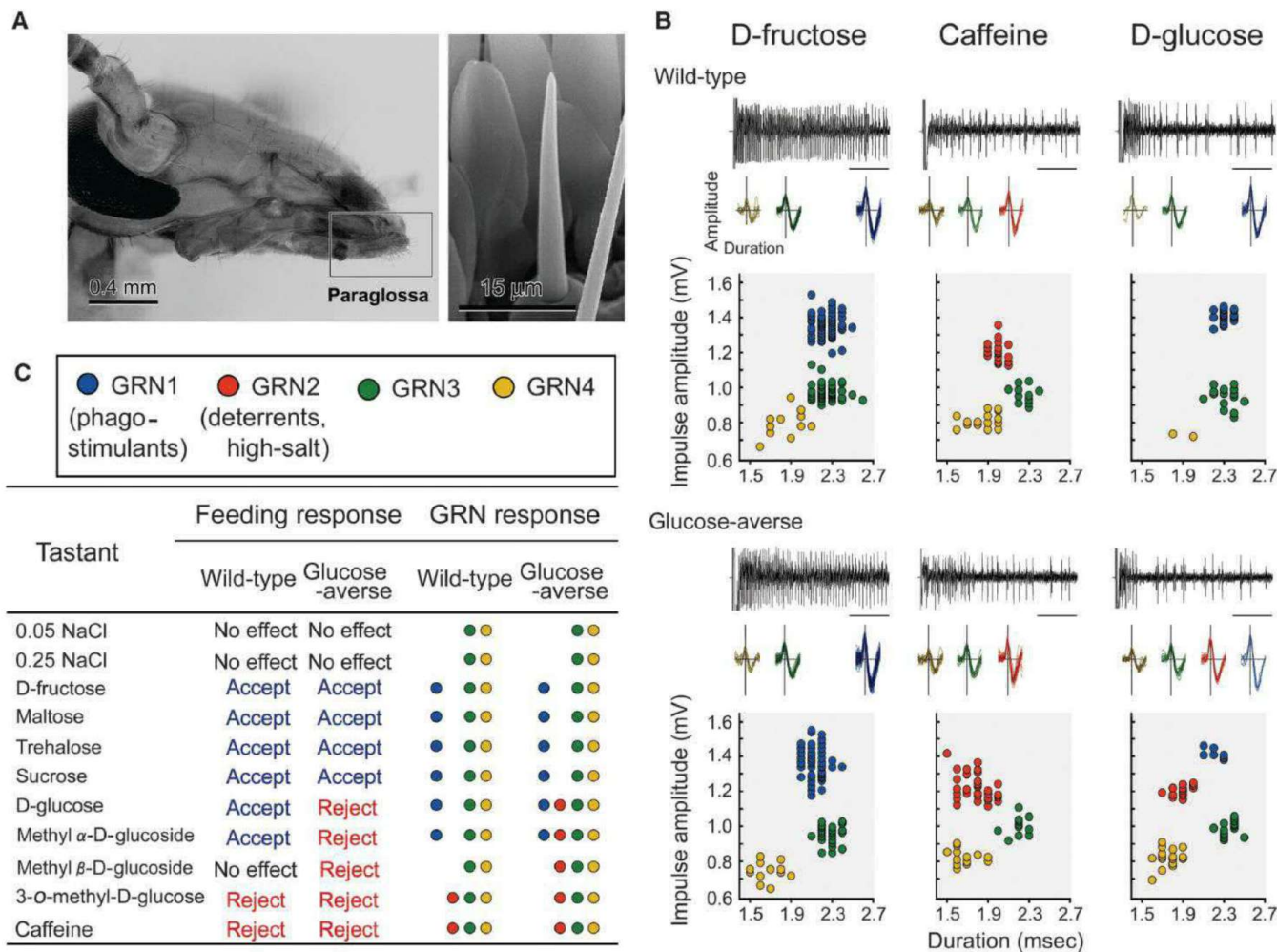


Fig. 1. Sensitivities of the GRNs of wild-type (WT) and GA (T164-BC) cockroaches to various tastants. (A) Side view of the right paraglossa of a WT male cockroach (left, maxillary and labial palps were removed), and a taste sensillum used in electrophysiological recordings (right). (B) GRN responses, showing sample recordings (top) of the same sensillum stimulated sequentially with fructose, caffeine, and glucose (top); impulse sorting (middle); and hier-

archical cluster analysis (bottom). The time bar under each recording indicates 200 ms. (C) Responsiveness of GRNs of WT and GA cockroaches (20 sensilla each) to 10 tastants. Feeding responses are from fig. S3. Fructose elicited impulses in GRN1, and caffeine elicited impulses in GRN2 in both strains. Glucose and related compounds stimulated GRN1 in WT cockroaches and both GRN1 and GRN2 in GA cockroaches.

new behavioral trait—glucose aversion. Glucose-averse (GA) cockroaches avoid eating glucose-containing baits (movies S1 to 4 and fig. S1), resulting in failure of otherwise highly effective baits (7). The GA trait is heritable (7, 8), and the aversive response is robustly evoked by glucose alone (7, 9). Although growth and reproduction are slower in GA than in wild-type cockroaches (10), GA cockroaches outcompete wild-type cockroaches under the strong selection pressure of glucose-containing baits (7, 11).

We hypothesized that the GA trait could be encoded by changes in glucose detection. Tastant detection in insects occurs in peripheral gustatory receptor neurons (GRNs), which are housed within hairlike sensilla on the mouthparts (12, 13). The GRNs have modal taste specificity, so in *Drosophila*, for example, four GRNs encode four taste classes: sugar-, bitter-, water- and salt-sensitive GRNs (13, 14). Each GRN expresses multiple

gustatory receptors (GRs) that recognize tastants and transduce information about their quality and strength into neuronal impulses that can be distinguished by their amplitude and duration (15, 16). As in other animals, tastants that activate sugar-GRNs elicit appetitive behavior (13, 17) and tastants that activate bitter-GRNs drive aversive behavior (13, 18).

The organization and functions of GRNs in the German cockroach are poorly understood (19). We concentrated on glucose-sensitive sensilla on the paraglossae (Fig. 1A) because the paraglossae alone can drive glucose acceptance in wild-type cockroaches and its rejection in GA cockroaches (9). Analysis of impulse waveforms [Fig. 1B; also see (20)] and cross-adaptation experiments (fig. S2) in wild-type cockroaches demonstrated that glucose-sensitive sensilla contain four distinct GRNs. Fructose and glucose selectively stimulated GRN1, whereas caffeine selectively stimulated GRN2.

GRN3 and GRN4 responded to both sugars and caffeine. Using a panel of tastants (Fig. 1C and fig. S3), we established that all tastants that stimulated feeding in wild-type cockroaches also stimulated GRN1 but not GRN2, and all deterrents stimulated GRN2 but not GRN1. The results indicate that the appetitive and aversive inputs in wild-type cockroaches segregate by the organization of GRN1 (sugar-GRN) and GRN2 (bitter-GRN) at the peripheral sensory level, as in other insect species (12, 13, 19).

The sugar- and bitter-GRN sensitivities of GA cockroaches (strain T164-BC) were considerably different from those of wild-type cockroaches. Glucose stimulated four rather than only three types of GRNs (Fig. 1B and fig. S2), corresponding to the sugar-GRN, bitter-GRN, GRN3, and GRN4 of wild-type cockroaches. Electrophysiological recordings from GA cockroaches with 10 tastants further demonstrated that the bitter-GRN

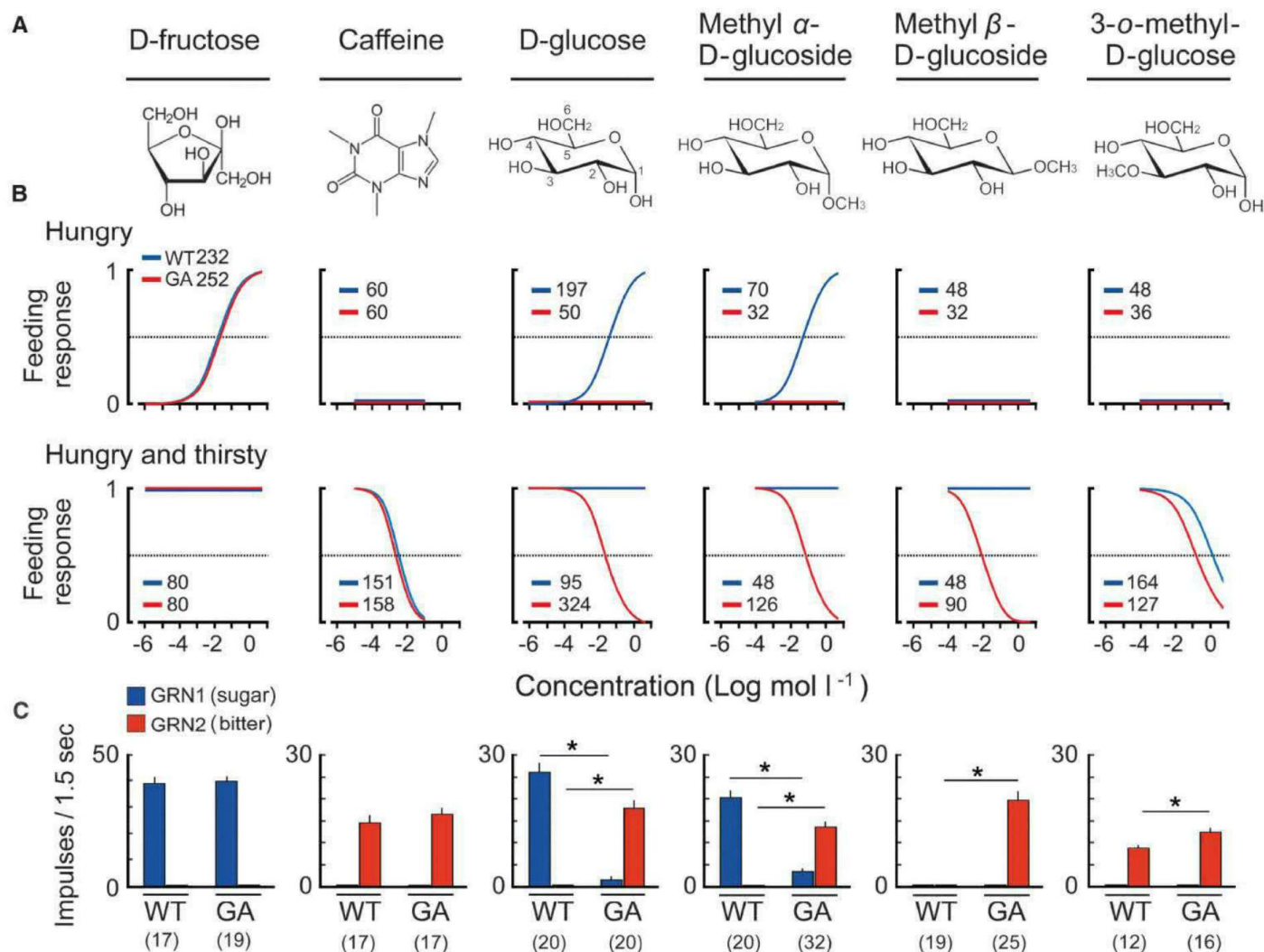


Fig. 2. Behavioral and electrophysiological responses to six tastants. (A) Chemical structures of tastants. (B) Dose-feeding responses in WT (blue) and GA (T164-BC, red) cockroaches motivated to accept phagostimulants but not water (Hungry), or to take both phagostimulants and water (Hungry and thirsty). Feeding response is the proportion of cock-

roaches ingesting the test solution, and the legends indicate sample size. GA cockroaches rejected glucose and related compounds. (C) The sugar- and bitter-GRNs of WT and GA cockroaches respond differentially to six tastants (mean $\pm$ SEM). Number of tested sensilla is in parentheses. (* $P < 0.05$, Student's t test).

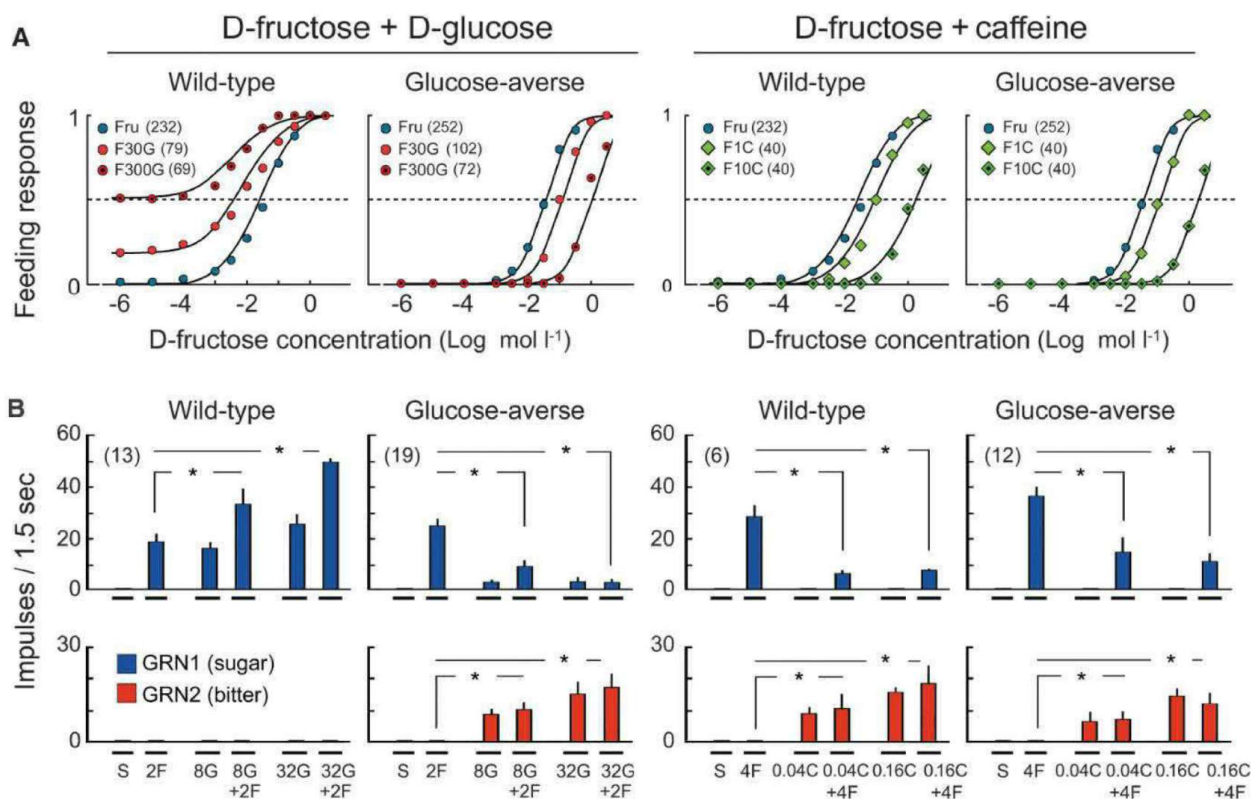


Fig. 3. Glucose aversion is elicited by stimulation of bitter-GRNs and inhibition of sugar-GRNs. (A) Cockroaches were tested with fructose alone (Fru), fructose mixed with 30 or 300 mmol liter⁻¹ glucose (F30G and F300G), and fructose mixed with 1 or 10 mmol liter⁻¹ caffeine (F1C and F10C). Numbers of tested WT and GA (T164-BC) cockroaches are in the legends (in parentheses). The response to fructose alone is also in Fig. 2B. **(B)** Sensitivity of sugar-GRN (top, blue) and bitter-GRN (bottom, red) to fructose alone and to

binary mixtures (means $\pm$ SEM). S, 0.25 mmol liter⁻¹ NaCl (control electrolyte); 2F and 4F, 2 and 4 mmol liter⁻¹ fructose; 8G and 32G, 8 and 32 mmol liter⁻¹ glucose; 0.04C and 0.16C, 0.04 and 0.16 mmol liter⁻¹ caffeine. Number of tested sensilla is in parentheses. The GRN responses to fructose alone were compared to the responses to binary mixtures (analysis of variance, Dunnett's test, $*P < 0.05$). Glucose and caffeine attenuate the feeding response to fructose in GA cockroaches and depress the sugar-GRN responses.

responded to glucose and all the tastants that elicited aversive behavior (Fig. 1C and fig. S3). We therefore suggest that glucose and related compounds drive the aversive response in GA cockroaches by stimulating the bitter-GRN, the same GRN that is stimulated by caffeine in both cockroach strains (Fig. 1C). By contrast, GRN3 and GRN4 responded without any apparent discrimination among stimuli (Fig. 1C, fig. S4A, and table S1), suggesting that they do not contribute to the differential discrimination of appetitive and aversive tastants by the two cockroach strains.

We compared the sensitivities of the sugar- and bitter-GRNs in the wild-type and GA strains with dose-behavioral response studies with six tastants (Fig. 2A). The two cockroach strains showed similar behavioral and GRN responses to fructose and caffeine (Fig. 2, B and C), suggesting that wild-type and GA cockroaches have fundamentally similar gustatory neural networks for appetitive and aversive behaviors. However, glucose and two related compounds stimulated the bitter-GRN in GA cockroaches (Fig. 2, B and C), and 3-*o*-methyl-D-glucose, which was aversive to both strains, elicited significantly higher bitter-GRN responses in GA than in wild-type cockroaches.

The results suggest that in wild-type cockroaches, glucose and related compounds are discriminated structurally by narrowly tuned receptors on sugar-GRNs, eliciting appetitive behavior. In GA cockroaches, by contrast, the expression of a broadly tuned receptor or multiple narrowly tuned receptors may contribute to the broad acceptance of glucose and related compounds by bitter-GRNs, driving aversive behavior.

Sugar-GRNs in GA cockroaches also exhibited a significantly lower response to glucose than in wild-type cockroaches (Fig. 2C). We tested whether the sugar-GRNs of GA cockroaches are less sensitive to glucose, or if their responses are depressed by the activities of adjacent GRNs. Complementary behavioral assays and electrophysiological recordings with mixtures of phagostimulants and deterrents revealed that in GA cockroaches, both glucose and caffeine attenuated the appetitive response to fructose (Fig. 3A and table S2) and significantly depressed the sugar-GRN responses relative to fructose alone (Fig. 3B). By contrast, in wild-type cockroaches, combining glucose with fructose increased both the appetitive response and the electrophysiological responses of sugar-GRNs compared to fructose alone (Fig. 3B). These results demonstrate that GA cockroaches

detect glucose as a genuine deterrent, which also suppresses sugar-GRN responses, as alkaloids and glucosides do in other insect species (21–23).

How prevalent is this mechanism in glucose-averse field populations? We screened the feeding responses of 19 field-collected populations and found seven populations with GA cockroaches (Fig. 4A). Two of these strains were used in behavioral and GRN dose-response studies. Although both were less GA than the lab-selected strains (Fig. 4B and table S2), in both strains glucose stimulated the bitter-GRN (Fig. 4C) and depressed the sugar-GRN (table S1). In four GA strains, the behavioral feeding responses negatively correlated with bitter-GRN responses (Fig. 4D and table S3). The wild-type and field-collected strains did not differ in GRN sensitivities for both fructose and caffeine (fig. S5 and table S1), confirming that a similar mechanism gave rise to glucose aversion in multiple cockroach populations.

Most natural genetic polymorphisms in taste receptors modify behavioral responses over a finite range, from exquisite sensitivity to complete insensitivity to a particular tastant [e.g., (24)]. In bait-selected cockroach populations, however, the modal specificity of glucose has been dramatically

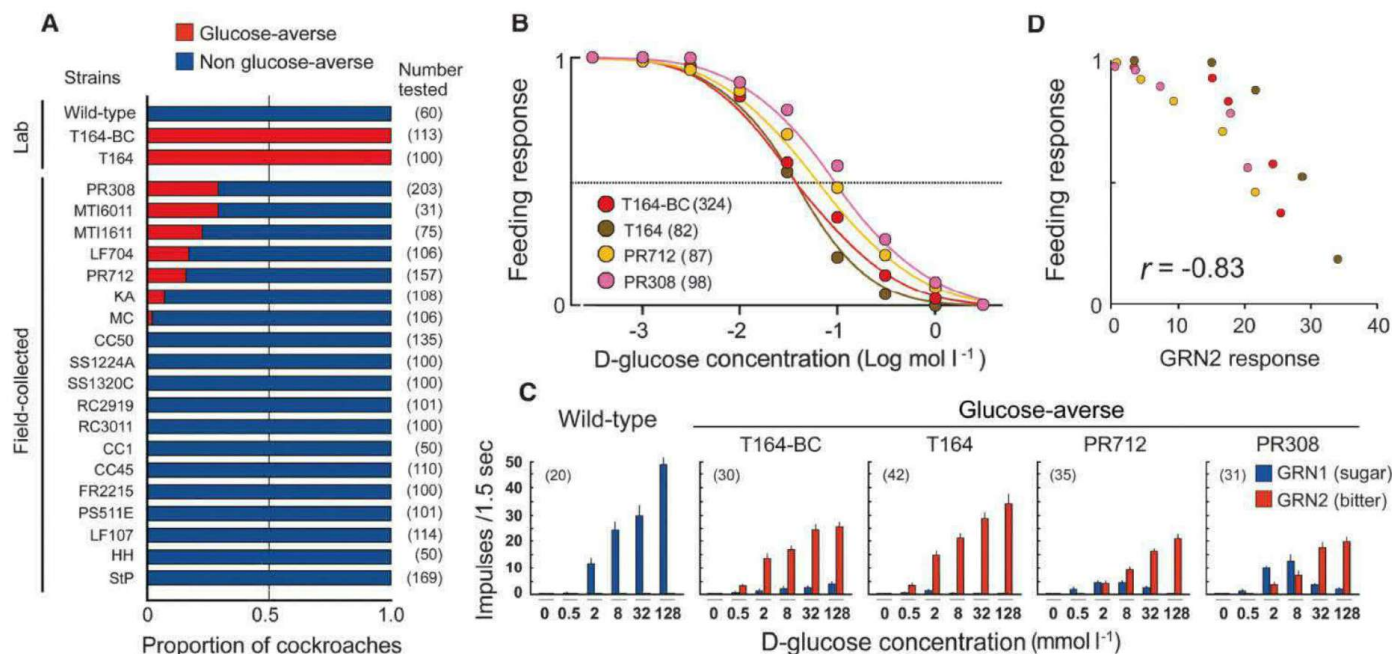


Fig. 4. Glucose stimulates bitter-GRNs in field-collected cockroaches. (A) Behavioral assays showing 7 of 19 field-collected populations with some GA cockroaches. (B) Dose-feeding responses to glucose in four GA strains, with the number of tested cockroaches in parentheses. T164-BC response to glucose is also shown in Fig. 2B, and the median effective concentration (EC_{50})

for each strain is in table S2. (C) Dose-GRN responses to glucose in WT and four GA strains (mean impulse frequency $\pm$ SEM, with number of tested sensilla in parentheses). (D) Feeding responses [from (B)] and GRN2 responses [from (C)] at similar glucose concentrations are negatively correlated (r , Pearson's correlation coefficient, $P < 0.001$, table S3).

transformed from “sweet” and highly phagostimulatory to “bitter” and highly deterrent. Generally, bitter-GRNs of insects coexpress a large number of GRs (18, 25) and are therefore broadly tuned to respond to various deterrents (18, 21, 22). The coexpression patterns of GRs ultimately account for the unique sensitivity of bitter-GRNs and their capacity to selectively respond to specific deterrents (18). Our electrophysiological studies with GA cockroaches suggest two major hypotheses: One or more mutations have either (i) modified the structure of GRs on the bitter-GRN to accept glucose and/or (ii) caused the misexpression of native glucose GRs on the bitter-GRN. The structure-activity studies tentatively support the former hypothesis that the glucose-sensitive GRs on bitter-GRNs are differently tuned from the native glucose GRs on sugar-GRNs, because wild-type and GA cockroaches responded differently—both behaviorally and with GRN responses—to changes in the chemical structures of glucose and related compounds.

Our results show that by recruiting glucose and related sugars as bitter-GRN ligands, a gain-of-function adaptation has emerged, expressing glucose-aversion as a novel behavior that offers protection against toxic baits. The change in valence of glucose, without compromising the exquisite sensitivity of the gustatory system to glucose, highlights the specificity of this adaptive change. Moreover, the aversion to glucose is further amplified by a preexisting inhibition of sugar-GRN responses by deterrents. Glucose aversion is a

clear example of a chemosensory gain-of-function adaptation that confers behavioral resistance to anthropogenic pressures, protecting the German cockroach from insecticides.

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Supplementary Materials

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Movies S1 to S4

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Ribosomal Protein SA Haploinsufficiency in Humans with Isolated Congenital Asplenia

Alexandre Bolze,^{1,2†} Nizar Mahlaoui,³ Minji Byun,¹ Bridget Turner,⁴ Nikolaus Trede,⁴ Steven R. Ellis,⁵ Avinash Abhyankar,¹ Yuval Itan,¹ Etienne Patin,⁶ Samuel Brebner,¹ Paul Sackstein,¹ Anne Puel,^{2,7} Capucine Picard,^{2,7,8} Laurent Abel,^{1,2,7} Lluís Quintana-Murci,⁶ Saul N. Faust,^{9,10*} Anthony P. Williams,^{10,11*} Richard Baretto,^{12*} Michael Duddridge,^{12*} Usha Kini,^{13*} Andrew J. Pollard,^{14*} Catherine Gaud,^{15*} Pierre Frange,^{16,17*} Daniel Orbach,^{18*} Jean-François Emile,^{19*} Jean-Louis Stephan,^{20*} Ricardo Sorensen,^{21*} Alessandro Plebani,^{22*} Lennart Hammarstrom,^{23*} Mary Ellen Conley,²⁴ Licia Sella,²⁵ Jean-Laurent Casanova^{1,2,7,16}

Isolated congenital asplenia (ICA) is characterized by the absence of a spleen at birth in individuals with no other developmental defects. The patients are prone to life-threatening bacterial infections. The unbiased analysis of exomes revealed heterozygous mutations in *RPSA* in 18 patients from eight kindreds, corresponding to more than half the patients and over one-third of the kindreds studied. The clinical penetrance in these kindreds is complete. Expression studies indicated that the mutations carried by the patients—a nonsense mutation, a frameshift duplication, and five different missense mutations—cause autosomal dominant ICA by haploinsufficiency. *RPSA* encodes ribosomal protein SA, a component of the small subunit of the ribosome. This discovery establishes an essential role for *RPSA* in human spleen development.

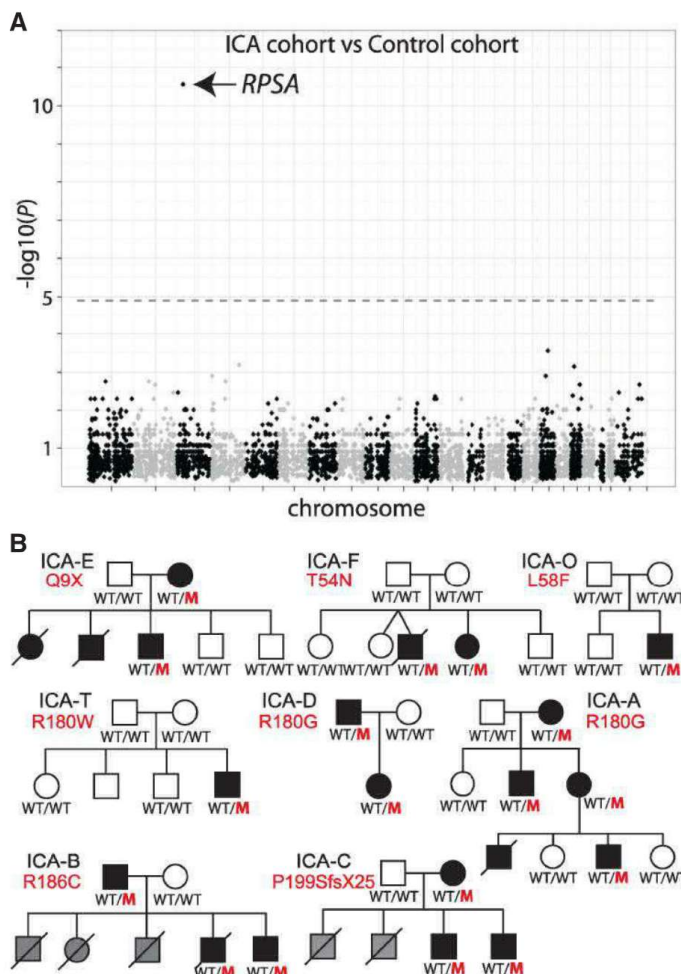
Patients with isolated congenital asplenia (ICA) are born without a spleen and display no other known developmental anomalies [Mendelian Inheritance in Man (MIM) code 271400] (*1–3*). Only 73 patients from 48 kindreds have been reported to date (*1, 3–6*). We

recruited an international cohort of 33 ICA patients from 23 kindreds (fig. S1 and table S1). Most patients with ICA, particularly the index cases, died in childhood from invasive bacterial disease (*1*). Because of the high proportion of familial cases (*1*), we hypothesized that ICA might result from single-gene inborn errors of spleen development. Moreover, ICA seems to segregate as an autosomal dominant (AD) trait in five multiplex kindreds (A to E in fig. S1). We have reported a candidate heterozygous mutation in *NKX2-5* in one kindred with AD ICA (*7*), but the genetic etiology of ICA remains essentially unknown. We therefore set out to decipher the main genetic etiology of ICA, both to cast light on the development of the human spleen and to guide clinical care and genetic counseling in families with ICA.

Given the apparent clinical homogeneity of the ICA patients, we hypothesized that there would be at least some genetic homogeneity among the 23 kindreds studied. We therefore sequenced one exome (*8–11*) from each of the 23 kindreds,

Fig. 1. *RPSA* heterozygous coding mutations are the most frequent genetic etiology of ICA. (A) Manhattan plot showing the *P*-value for tests of the hypothesis that “mutations in a given gene were not specific to the ICA cohort.” Each dot represents one gene.

x axis: Physical position of each gene on the chromosome. y axis: $-\log_{10}(P)$. *P* was calculated for Fisher’s exact test comparing 23 exomes from 23 ICA kindreds and 508 exomes from patients with phenotypes other than invasive bacterial disease. The gray dashed line indicates threshold for statistical significance ($0.05/4,222 = 1.2 \times 10^{-5}$). (B) Familial segregation of all *RPSA* coding mutations. Mutations are described in red. Capital letters represent the kindred code. When available, the genotype of *RPSA* is indicated under each symbol. M, mutant. Black, ICA; gray, probable ICA.



¹St. Giles Laboratory of Human Genetics of Infectious Diseases, Rockefeller University, New York, NY 10065, USA. ²University Paris Descartes, Sorbonne Paris Cité, Imagine Institute, 75006 Paris, France. ³Pediatric Hematology-Immunology and Rheumatology Unit, French National Reference Center for Primary Immune Deficiencies (CEREDIH), Necker-Enfants Malades Hospital, 75015 Paris, France. ⁴The Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84112, USA. ⁵Department of Biochemistry and Molecular Biology, University of Louisville, Louisville, KY 40202, USA. ⁶Unit of Human Evolutionary Genetics, CNRS URA3012, Department of Genomes and Genetics, Pasteur Institute, 75015 Paris, France. ⁷Laboratory of Human Genetics of Infectious Diseases, Necker Branch, INSERM U980, 75015 Paris, France. ⁸Study Center of Primary Immunodeficiency, Necker-Enfants Malades Hospital, Assistance-Publique Hopitaux de Paris, 75015 Paris, France. ⁹NIHR Wellcome Trust Clinical Research Facility, University Hospital Southampton NHS Foundation Trust, Southampton SO16 6YD, UK. ¹⁰Faculty of Medicine and Institute for Life Sciences, University of Southampton, Southampton SO16 6YD, UK. ¹¹University Hospital Southampton NHS Foundation Trust, Southampton SO16 6YD, UK. ¹²Department of Immunology, University Hospitals Leicester NHS Trust, Leicester LE1 5WW, UK. ¹³Department of Clinical Genetics, Oxford University Hospitals NHS Trust, Oxford OX3 7LE, UK. ¹⁴Department of Pediatrics, University of Oxford, and the NIHR Oxford Biomedical Research Centre, Oxford OX3 9DU, UK. ¹⁵Department of Clinical Immunology, CHU Reunion Site Nord, 97405 Saint-Denis, Reunion Island, France. ¹⁶Pediatric Immunology-Hematology Unit, Necker-Enfants Malades Hospital, Assistance-Publique Hopitaux de Paris, 75015 Paris, France. ¹⁷EA 3620, University Paris Descartes, Sorbonne Paris Cité, 75015 Paris, France. ¹⁸Pediatric Department, Curie Institute, 75005 Paris, France. ¹⁹EA 4340, University Versailles SQY and Ambroise Pare Hospital, Assistance-Publique Hopitaux de Paris, 92104 Boulogne, France. ²⁰Department of Pediatrics, CHU Nord, 42055 Saint-Etienne, France. ²¹Department of Pediatrics, Louisiana State University Health Sciences Center, Jeffrey Modell Diagnostic Center for Primary Immunodeficiencies and Children’s Hospital, New Orleans, LA 70118, USA. ²²Department of Pediatrics and Institute for Molecular Medicine “A. Nocivelli,” University of Brescia, Spedali Civili di Brescia, 25123 Brescia, Italy. ²³Division of Clinical Immunology, Department of Laboratory Medicine, Karolinska Institutet at Karolinska University Hospital Huddinge, SE-14186 Stockholm, Sweden. ²⁴Department of Pediatrics, University of Tennessee College of Medicine, Memphis, TN 38101, USA. ²⁵Department of Cell and Developmental Biology, Weill Medical College of Cornell University, New York, NY 10065, USA.

*These authors contributed equally to this work.

†Corresponding author. E-mail: albo719@rockefeller.edu; alexandre.bolze@gmail.com

including the kindred bearing the *NKX2-5* mutation, and analyzed them together (fig. S1 and table S2). We hypothesized that the disease-causing variants would be very rare, because of the rarity of ICA (1). We also gave priority to coding mutations predicted not to be silent (nonsynonymous). We found that 764 genes in at least two ICA kindreds carried very rare and nonsynonymous mutations (table S3). We performed the same analysis on 508 control exomes sequenced in-house (table S4), to identify the best candidate morbid gene for ICA. We then used the results of these two analyses (comparison of ICA and controls) to test the null hypothesis that mutations in a given gene were not specific to ICA, by calculating the *P*-value for each gene in Fisher's exact test. *RPSA* had a highly significant *P*-value of 2.89×10^{-11} (Fig. 1A), which indicated that mutations in this gene were specific to the ICA cohort. The coding region of *RPSA* carried very rare non-

synonymous variants in eight of 23 ICA kindreds and in only one of the 508 control exomes. No other gene had a statistically significant *P*-value (table S5).

RPSA encodes the ribosomal protein (RP) SA. The genes encoding RPs have numerous pseudogenes (12), which can hinder their sequencing. *RPSA* has 61 processed pseudogenes (table S6) (12). We thus Sanger sequenced all coding exons of *RPSA* in all 33 ICA patients, using primers mapping to the introns of *RPSA*, which cannot amplify *RPSA* pseudogenes (13). Eighteen of the 33 patients (55%) had *RPSA* mutations (Fig. 1B and fig. S2). Altogether, we identified seven mutations in eight kindreds: one nonsense mutation [Gln⁹ replaced by a termination codon (p.Q9X)], one frameshift duplication (p.P199SfsX25), and five missense mutations, including the recurrent Gly substitution for Arg¹⁸⁰ (p.R180G) (table S7). A missense mutation, Val substitution for Met¹⁸⁵

(p.M185V), was identified in one control exome from a patient displaying severe viral infection, but not ICA. The seven ICA mutations were not present in more than 10,000 alleles reported in public databases (table S8). Moreover, the five ICA-associated missense mutations affected residues strictly conserved in mammals, vertebrates, and even yeast (fig. S3). All ICA patients in these eight kindreds carried a mutation in *RPSA*, and all individuals carrying *RPSA* mutations displayed ICA (Fig. 1B).

It was striking that neither of the two parents carried an *RPSA* mutation in kindreds F, O, and T, although a mutation was found in the two affected siblings in kindred F and in the sporadic patients in kindreds O and T (Fig. 1B). Microsatellite analysis confirmed the parental relationships of the samples (table S9 and fig. S4). Thus, mutations in kindreds O and T appeared de novo and resulted from a germline mosaicism in kindred

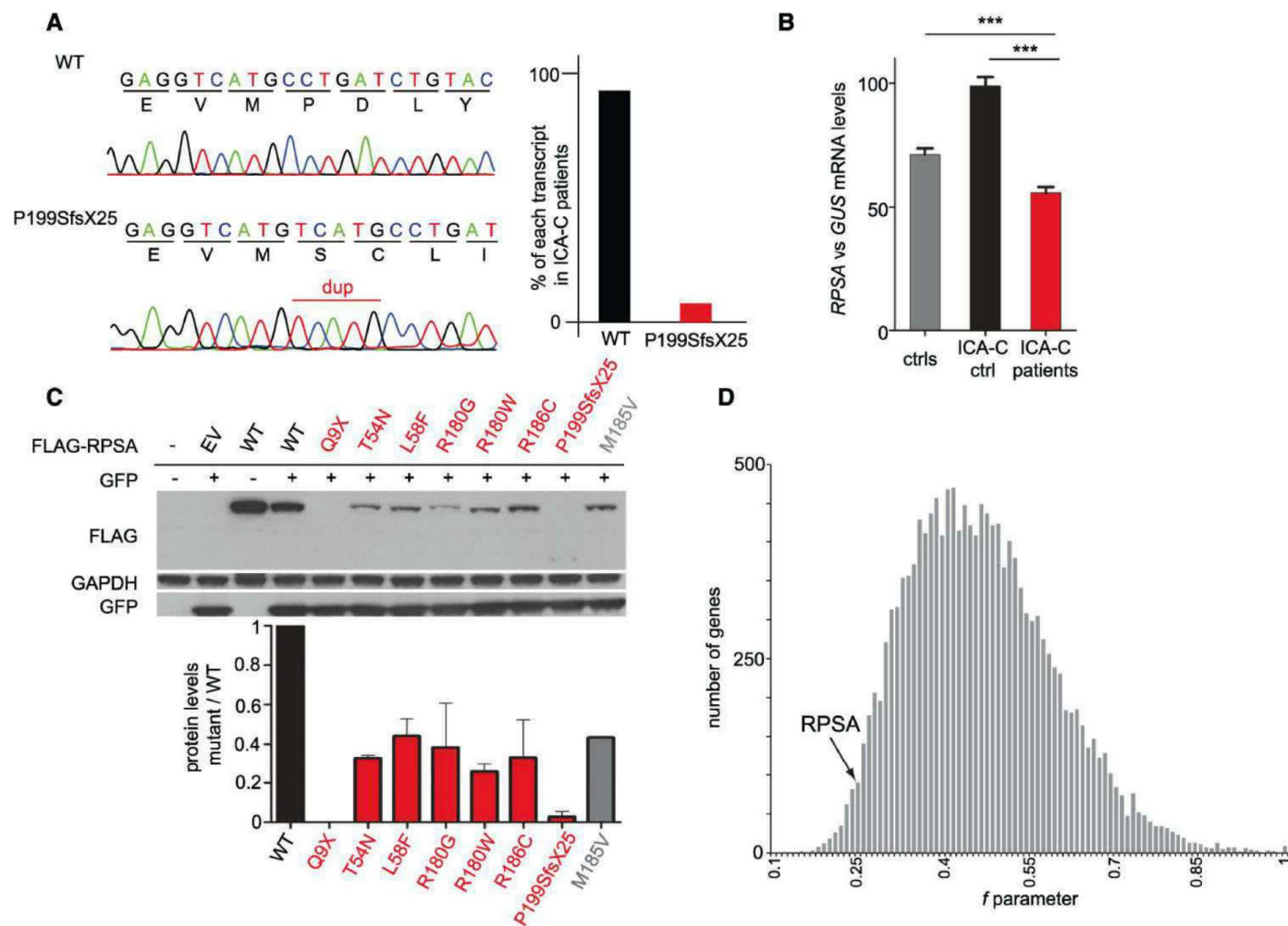


Fig. 2. Haploinsufficiency at the *RPSA* locus. (A) *RPSA* cDNA was obtained from activated T cells of patients ICA-C-I.2, ICA-C-II.3, and ICA-C-II.4. Sequences of WT and mutant cDNA are shown. The deduced frequency of each mRNA is indicated in the diagram on the right. (B) Relative levels of *RPSA* mRNA in activated T cells from three patients, a healthy member of kindred C (ICA-C-I.1), and four unrelated healthy controls. Peripheral blood mononuclear cells were activated with phytohemagglutinin for 5 days. A mean of four independent experiments is shown. Error bars indicate the SEM. ****P* < 0.001. (C) Immunoblot

showing the levels of the WT and mutant *RPSA* proteins following overproduction in HEK293T cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), loading control; green fluorescent protein (GFP), transfection control. The blot shown is representative of four independent experiments. (Bottom) Intensity of the bands corresponding to the FLAG antibody normalized with respect to the band from the GFP immunoblot. Error bars indicate the SEM. (D) Genome-wide distribution of the strength of purifying selection acting in 14,993 human genes. A low *f* estimate (13) indicates that the gene is particularly constrained.

F. Moreover, a comparison of the haplotypes at the *RPSA* locus between patients from families A and D showed that the p.R180G mutation was not inherited from a common ancestor (a founder effect), but had instead occurred twice, independently (fig. S5). This is consistent with the complete penetrance of *RPSA* mutations for ICA and the high mortality of ICA, because a founder effect would require the existence of multiple generations of healthy *RPSA* heterozygotes (fig. S5), before the advent of antibiotics. Collectively, these genetic results suggest that heterozygous coding mutations in *RPSA* underlie most cases of ICA, with apparently complete clinical penetrance. In particular, heterozygous coding mutations in *RPSA* were found to underlie ICA in all multiplex kindreds displaying an AD pattern of inheritance studied, including the kindred with the heterozygous mutation in *NKX2-5* [ICA-B (7)].

Our identification of two mutations resulting in a premature termination codon (p.Q9X and p.P199SfsX25) led us to hypothesize that haploinsufficiency at the *RPSA* locus might underlie AD ICA. Cloning and analysis of cDNA generated from activated T cells of three patients from family C showed that less than 10% (12 out of 160) of the transcripts carried the p.P199SfsX25 mutation (Fig. 2A), which suggested that the mRNAs generated from the mutated allele were subject to nonsense-mediated mRNA decay (fig. S6). *RPSA* mRNA levels in activated T cells from these patients were only half those in their healthy relative (Fig. 2B). We then investigated the missense mutations, by overproducing the N-terminally FLAG-tagged mutant and wild-type (WT) proteins in human embryonic kidney–293T (HEK293T) cells. The mutant proteins were produced in much smaller amounts than the WT protein (Fig. 2C). We next determined whether *RPSA* was under purifying selection in the general population. *RPSA* is at the 2.8th percentile with respect to a metric of purifying selection (Fig. 2D) (14) among ~15,000 genes exome-sequenced by the 1000 Genomes Project (15). These data suggest that heterozygosity for null *RPSA* alleles underlies AD ICA and possibly accounts for the strong purifying selection acting on these alleles in the population.

It is surprising that germline mutations in *RPSA* cause a spleen-specific developmental defect. *RPSA* is ubiquitously expressed. *RPSA* is involved in pre-ribosomal RNA (pre-rRNA) processing (16) and is part of the small subunit of the ribosome (17). *RPSA* was not known to be involved in spleen development, which is controlled by a cascade of transcription factors (e.g., *Tlx1*, *Nkx2-5*, and *Wt1*) in mice (7, 18). Moreover, haploinsufficiency of any of 10 other RPs, including RPS19, is associated with Diamond-Blackfan anemia (DBA), which is characterized by bone marrow failure and a broad range of developmental defects, ranging from craniofacial defects to thumb abnormalities (19–21). Patients with *RPSA* mutations present none of these phenotypes (table S10). Conversely, DBA patients mutated in other RPs display no spleen abnormalities. At the cellular level, there

was no pre-rRNA processing defect in activated lymphocytes from *RPSA*-mutated ICA patients (fig. S7), in contrast with the pre-rRNA-processing defects observed in all RP-mutated DBA patients (20). Last, heterozygosity for a null *Rpsa* allele in the mouse is not associated with ICA (fig. S8 and table S11). We do not yet understand the pathogenesis of ICA. However, the emerging idea that ribosomes can be “specialized” might account for the narrow phenotype caused by mutations in *RPSA* (22). Alternatively, an extra-ribosomal function of *RPSA* (23) may explain the phenotype. The surprising connection between *RPSA* and spleen development in humans calls for explorations of the underlying mechanisms.

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Supplementary Materials

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Futile Protein Folding Cycles in the ER Are Terminated by the Unfolded Protein O-Mannosylation Pathway

Chengchao Xu,^{1,2} Songyu Wang,^{1,2*} Guillaume Thibault,¹ Davis T. W. Ng^{1,2,3,†}

Newly synthesized polypeptides fold and assemble with assistance from protein chaperones. Full maturation can take multiple attempts, exchanging chaperones at each round. Improperly folded molecules must exit folding cycles and be degraded. In the endoplasmic reticulum (ER), prolonged substrate cycling is detrimental because it expends chaperone and energy resources and increases toxic reactive oxygen species. In budding yeast, we found that unfolded protein O-mannosylation terminated failed folding attempts through the Pmt1/Pmt2 complex. O-mannosylation incapacitated target molecule folding and removed them from folding cycles by reducing engagement with the Kar2 chaperone. In an in vitro protein refolding assay, the modification intrinsically and irreversibly disabled the folding potential of the substrate. Thus, protein folding termination can involve a covalent glycosylation event.

Nascent polypeptides emerge from ribosomes into the cytoplasm, a crowded environment of macromolecules that can interfere with the folding process (1). To optimize folding, chaperone systems reduce unfavorable interactions through cycles of binding and release. Unfolded proteins, after repeated folding attempts, are eliminated through protein quality control pathways. The decision to terminate folding is pivotal in the transition from the anabolic phase to ca-

tabolism. Futile protein folding cycles consume limited chaperone and energy resources, but what ends unproductive attempts remains unclear (2–5).

¹Temasek Life Sciences Laboratory, National University of Singapore, Singapore. ²Department of Biological Sciences, National University of Singapore, Singapore. ³Duke-National University of Singapore Graduate Medical School, Singapore.

*Present address: Howard Hughes Medical Institute and Department of Cell Biology, Harvard Medical School, USA. †Corresponding author. E-mail: davis@tll.org.sg

In the secretory pathway, protein synthesis and quality control functions are situated in the endoplasmic reticulum (ER). Proteins failing to fold properly are removed through ER-associated degradation (ERAD), the terminal phase of ER quality control (ERQC). A variety of folding-disabled proteins as models characterized the known ERAD pathways (6). To reveal earlier events of ERQC, we needed a folding-competent protein unable to complete its maturation within the constraints of ERQC. Such molecules should mimic futile folding cycles and be subject to termination mechanisms deployed. Folding competence provides a direct means to determine whether interference reflects a bona fide termination mechanism. Because proteins evolve in concert with their QC systems, suitable substrates must be created or sourced from exogenous systems. With this view, we considered ER-targeted green fluorescent protein (ER-GFP) as a candidate (fig. S1). GFP folds poorly in the mammalian ER (7). A similar folding deficiency was observed in yeast by expressing ER-GFP in the translocation mutant *sec63-1*. Diverted to the cytosol, ER-GFP fluoresced strongly compared with the

weak ER signal in wild-type cells (Fig. 1A). Although fluorescence is an indicator of GFP folding, we confirmed its poor folding in the ER using two additional assays. Disulfide-linked oligomers, a product of ER-GFP misfolding, formed as readily as the folding defective ER- Δ 2GFP variant (Fig. 1B and fig. S1) (7, 8). Secondly, ER-GFP displayed pronounced protease sensitivity similar to ER- Δ 2GFP, consistent with structural disorder (Fig. 1C).

The luminal ERAD pathway uses a glycan timing mechanism to select substrates (9, 10). The concept of a time constraint for folding might also apply here. GFP is a relatively slow folding protein, whose intrinsic rate might exceed the ERQC limit (11). To investigate this possibility, a fast-folding GFP variant called P7 was engineered like ER-GFP (fig. S1, ER-GFP^{fast}) (12). In wild-type cells, ER-GFP^{fast} localized to the ER, like ER-GFP, but emitted a much stronger fluorescence signal (Fig. 2A and fig. S1). The inability to form disulfide-linked oligomers and its strong resistance against protease confirmed better folding proficiency (Fig. 2, B and C). Thus, ER-GFP^{fast} was not subject to folding termination because of its faster folding rate.

The substrate pair ER-GFP and ER-GFP^{fast} appeared to provide a model that could delineate the early steps of ERQC. Another characteristic of ER-GFP, the lack of ERAD signals, increased its stability and, thus, its utility as a model by removing the need to account for turnover (fig. S2A). The data suggested that an active folding termination mechanism targeted slowly folding ER-GFP. Because ER-GFP expression did not affect the maturation of other proteins (fig. S2B), the mechanism probably acts directly on substrates instead of the general folding machinery. In addition, the inability of ER-GFP molecules to fold even after extended periods suggested that termination is irreversible (Fig. 1C and fig. S2A). What could mark slowly folding proteins for termination? An attractive possibility is a covalent modification of misfolded proteins (13–20), which are O-mannosylated at serine and threonine residues, a process catalyzed by Pmt1/Pmt2 protein complex (21). Although the complex also modifies normal proteins, its specific regulation by the unfolded protein response (UPR) suggests a role in ER quality control (21, 22). We wondered

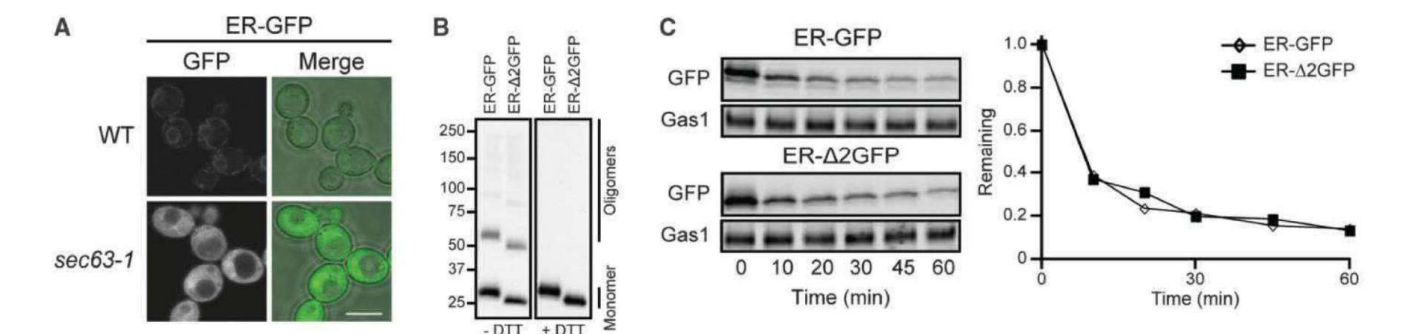


Fig. 1. ER-GFP misfolds in the ER. (A) Confocal images of cells expressing ER-GFP. Scale bar, 5 μ m. (B) Immunoblot for ER-GFP or ER- Δ 2GFP from wild-type (WT) extracts resolved in the absence (–DTT) or presence (+DTT) of reducing agent. (C) ER-GFP or ER- Δ 2GFP from WT cells treated with trypsin for time indicated.

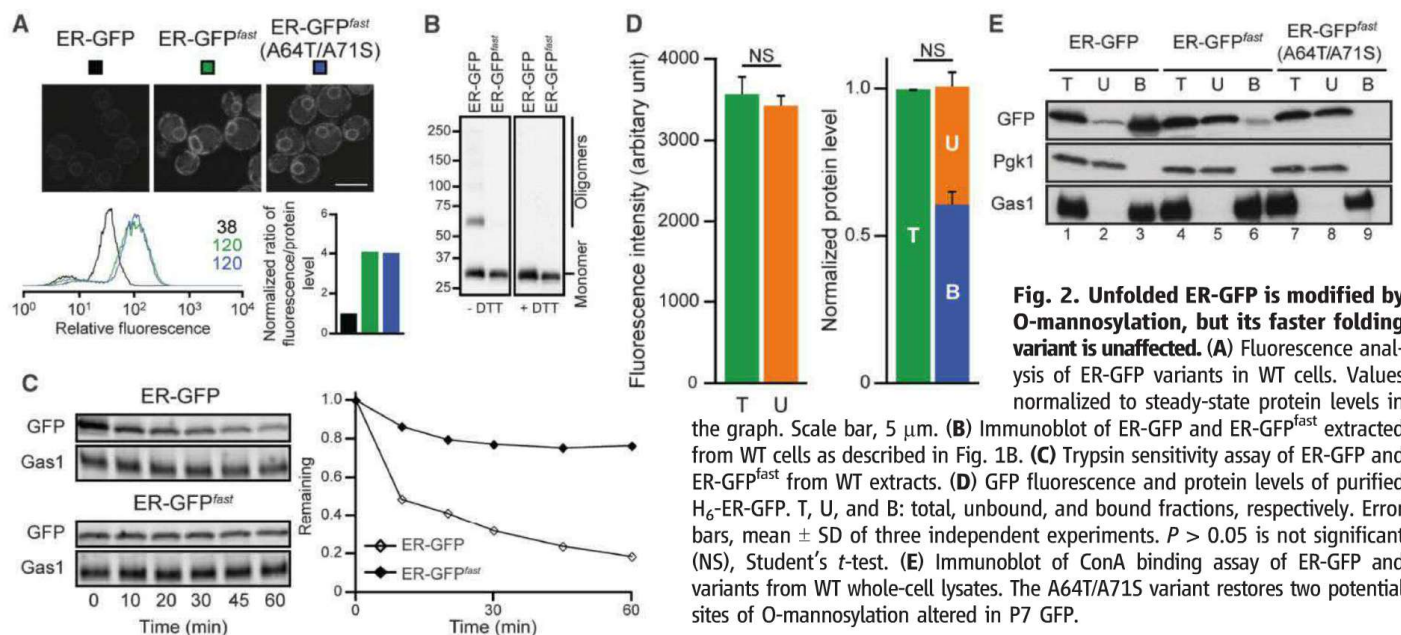
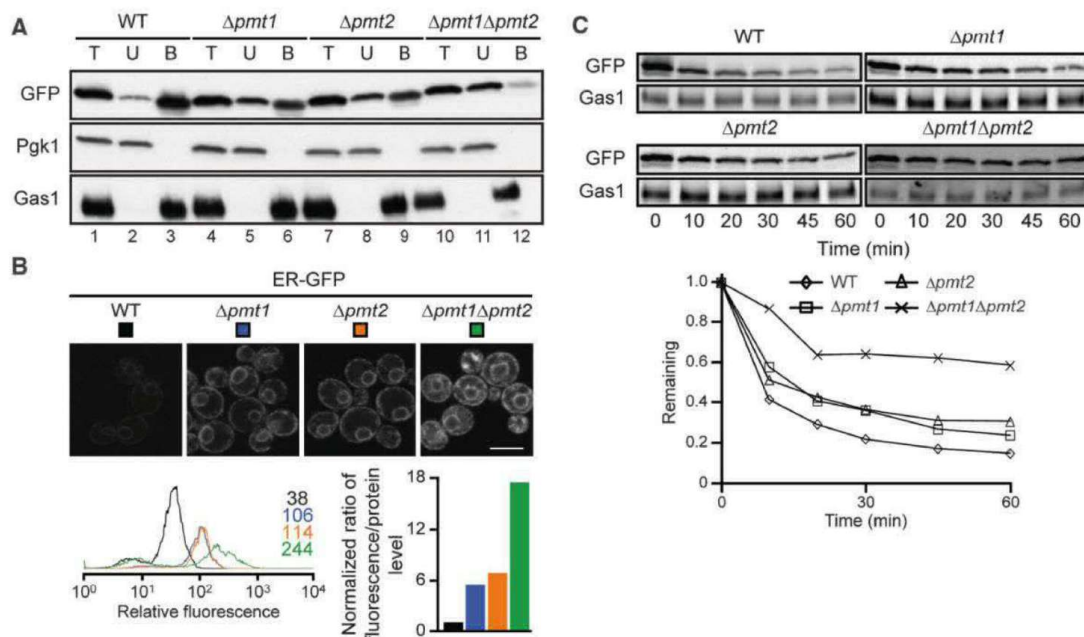


Fig. 2. Unfolded ER-GFP is modified by O-mannosylation, but its faster folding variant is unaffected. (A) Fluorescence analysis of ER-GFP variants in WT cells. Values normalized to steady-state protein levels in the graph. Scale bar, 5 μ m. (B) Immunoblot of ER-GFP and ER-GFP^{fast} extracted from WT cells as described in Fig. 1B. (C) Trypsin sensitivity assay of ER-GFP and ER-GFP^{fast} from WT extracts. (D) GFP fluorescence and protein levels of purified Hc-ER-GFP. T, U, and B: total, unbound, and bound fractions, respectively. Error bars, mean $\pm$ SD of three independent experiments. $P > 0.05$ is not significant (NS), Student's t -test. (E) Immunoblot of ConA binding assay of ER-GFP and variants from WT whole-cell lysates. The A64T/A71S variant restores two potential sites of O-mannosylation altered in P7 GFP.

Fig. 3. Unfolded protein O-mannosylation by Pmt1/Pmt2 actively terminates folding.

(A) Immunoblot of ConA binding assay of ER-GFP from WT and *pmt* mutant extracts. (B) Fluorescence analysis of WT and *pmt* mutant cells expressing ER-GFP by flow cytometry and plot of values normalized to steady-state protein levels. Scale bar, 5 μ m. (C) Trypsin sensitivity assay of ER-GFP from WT and *pmt* mutant cell extracts.



whether modifying small side chains of folding polypeptides with bulky hydrophilic mannose moieties could directly terminate folding.

To investigate our hypothesis, ER-GFP containing a six-histidine tag was purified from wild-type extracts and applied to immobilized Concanavalin A (ConA) (fig. S3A). ConA affinity chromatography is a classical and reliable method to separate glycosylated proteins from nonglycosylated proteins (23). ER-GFP lacks N-linked glycans, so any binding is due to O-glycans (fig. S1B) (24). The unbound fractions were collected and pooled, and the fluorescence was measured against the total fraction. Unfortunately, the bound fraction could not be completely eluted under native conditions because of tight binding. However, nearly all fluorescence activity was recovered in the unbound fraction even though the majority of the loaded material was bound to the ConA matrix (Fig. 2D and fig. S3B). Similar results were obtained using untagged ER-GFP directly from crude microsomal extracts (fig. S4). We next determined the extent of O-mannosylation on ER-GFP^{fast}. Unlike ER-GFP, ER-GFP^{fast} was found mostly in the unbound fraction (Fig. 2E). Because these molecules are nearly identical except for folding rate, the data are consistent with previous reports that unfolded protein O-mannosylation occurs after prolonged substrate occupancy in the ER (14, 16). Indeed, direct analysis using galactose-inducible promoters showed that ER-GFP was modified posttranslationally, whereas ER-GFP^{fast} remained mostly unmodified even after a prolonged chase (fig. S5). Thus, unfolded molecules of ER-GFP were O-mannosylated and folded proteins escaped modification.

It appears that ERQC could terminate folding midstream because ER-GFP folding is slower than cell-defined parameters. Alternatively, if ER-GFP inherently folds poorly, O-mannosylation could be the consequence of folding failure rather than

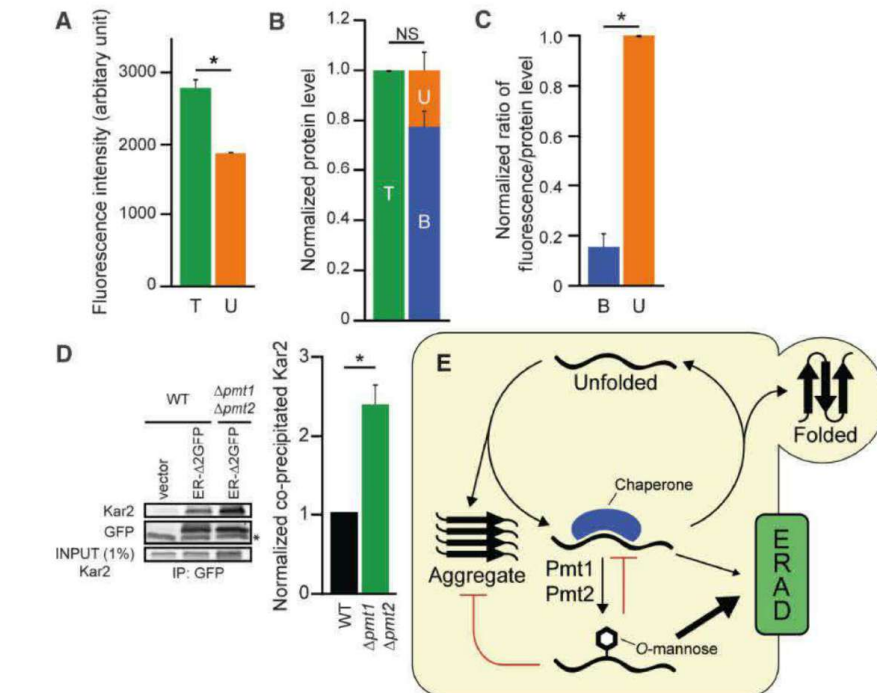


Fig. 4. Substrate O-mannosylation intrinsically impairs protein folding and reduces Kar2 association. (A and B) H₆-ER-GFP fluorescence and protein level of T, U, and B fractions after refolding. (C) H₆-ER-GFP fluorescence levels relative to protein levels after refolding. (D) Coimmunoprecipitation of ER- $\Delta 2$ GFP with Kar2 from WT or $\Delta pmt1\Delta pmt2$ extracts. Error bars, mean $\pm$ SD of three independent experiments. * $P < 0.01$ and $P > 0.05$ is not significant (NS), Student's *t* test. (E) Model of the UPOM mechanism in ERQC. Chaperones assist nascent polypeptides to fold in the ER. Proteins with low folding efficiency would be O-mannosylated by Pmt1/Pmt2. This modification prevents chaperone association and protein folding, inhibits aggregation, and promotes degradation.

the cause. To differentiate these possibilities, we analyzed ER-GFP folding in strains defective in the activity. If O-mannosylation specifically terminates folding, decreasing it should allow folding to proceed to completion. On the other hand, no

improvement would be expected if ER-GFP intrinsically folds poorly in the ER. ER-GFP extracted from $\Delta pmt1$ or $\Delta pmt2$ cells reduced ConA binding, whereas most binding was abolished from the double mutant (Fig. 3A). Next, ER-GFP fluorescence

was measured by flow cytometry and normalized to steady-state protein levels, and the relative intensities were plotted (Fig. 3B and fig. S6). Fluorescence levels were about six times as high in each single mutant and more than 15 times as high in the double mutant over wild type. ER-GFP protein extracted from *pmt* mutants displayed increased protease resistance and less disulfide-linked oligomer formation of similar magnitudes over wild type (Fig. 3C and fig. S7). $\Delta pmt1$ and $\Delta pmt2$ mutants induce the UPR, which might explain the folding improvement (25). However, the effect was not observed in a wide variety of mutants displaying a similarly elevated UPR or in wild-type cells expressing activated Hac1pⁱ transcription factor, which maximally induces the pathway (25, 26) (fig. S8).

We next determined whether O-mannosylation terminated folding by intrinsically altering substrate folding potential. For this, an *in vitro* refolding assay for ER-GFP was established. ER-GFP was purified from wild-type extracts, denatured, and allowed to refold during denaturant dilution by dialysis. Fluorescence activity was measured for half the total material. The other half was adsorbed to ConA Sepharose. The unbound fractions were pooled and fluorescence was measured. The unbound fraction represented 23% of the total protein but 67% of the fluorescence activity after refolding (Fig. 4, A and B, and fig. S9). Because the bound fraction could not be quantitatively eluted under native conditions, the maximum refolding potential of the O-mannosylated protein was about 15% of the unmodified fraction (Fig. 4C). This assay was not meant to replicate the environment of the ER but to measure the relative folding potential of these two forms. Thus, the *in vitro* conditions may support folding for a small fraction of the modified form that is not competent *in vivo*. Thus, substrate folding was directly disabled by O-mannosylation and, in the absence of a demannosylation mechanism, the termination of folding was effectively irreversible.

We hypothesize that an important function of a folding termination mechanism is to end futile chaperone-dependent folding cycles. To investigate this question, ER- $\Delta 2$ GFP expressed in wild-type and $\Delta pmt1 \Delta pmt2$ cells was assayed for Kar2 chaperone binding. Kar2 is the major protein-folding chaperone of the yeast ER (27). ER- $\Delta 2$ GFP was used so that an unfolded conformation could be maintained in both strains (fig. S10). More Kar2 is complexed with ER- $\Delta 2$ GFP in the mutant strain compared to wild type (Fig. 4D). Thus, O-mannosylation is used to terminate protein folding and take substrates out of nonproductive folding cycles.

Protein folding can involve multiple chaperone binding and release cycles (1, 2). In this vein, failure to fold due to sequence or stochastic abnormalities could lead to futile folding cycles that risk exhausting chaperone and energy resources. To avoid this scenario, nonproductive molecules must be taken out of folding cycles and degraded. Mechanisms of folding termination have been unclear. Interestingly, among several distinct ERAD pathways discovered in budding yeast (28), substrates from each

share the common link of being O-mannosylated (13–20). However, it does not appear to constitute part of a degradation signal because its requirement varies depending on the substrate (14–20). Our data provide a clear and unifying function for unfolded protein O-mannosylation (UPOM).

Protein folding is a fundamental cellular process, and its dysfunction underlies numerous human diseases. Here, we report a covalent mechanism, UPOM, used to terminate protein folding by setting a time limit (Fig. 4E). What constitutes and sets the “timer” remains unclear, but factors involved in protein folding are associated with the Pmt1/Pmt2 complex (19). Thus, substrate recognition could be through associated chaperones, by analogy to the Htm1/PDI complex in ERAD (10). Importantly, O-mannosylation inhibits protein aggregation and futile chaperone interactions (16, 18) (Fig. 4 and fig. S11). By using a bulky hydrophilic group as a modifier, the UPOM mechanism integrates several critical activities to neutralize aberrant folding products and maintain cellular protein homeostasis.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S11

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References (29–39)

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Identification of a Heteromeric Complex That Promotes DNA Replication Origin Firing in Human Cells

Dominik Boos, Mona Yekezare, John F. X. Diffley*

Treslin/TICRR (TopBP1-interacting, replication stimulating protein/TopBP1-interacting, checkpoint, and replication regulator), the human ortholog of the yeast Sld3 protein, is an essential DNA replication factor that is regulated by cyclin-dependent kinases and the DNA damage checkpoint. We identified MDM two binding protein (MTBP) as a factor that interacts with Treslin/TICRR throughout the cell cycle. We show that MTBP depletion by means of small interfering RNA inhibits DNA replication by preventing assembly of the CMG (Cdc45–MCM–GINS) holohelicase during origin firing. Although MTBP has been implicated in the function of the p53 tumor suppressor, we found MTBP is required for DNA replication irrespective of a cell's p53 status. We propose that MTBP acts with Treslin/TICRR to integrate signals from cell cycle and DNA damage response pathways to control the initiation of DNA replication in human cells.

Eukaryotic cells must ensure their entire genome is replicated exactly once in each cell cycle (1–3). The yeast Sld3 protein has emerged as a focal point of regulation of

replication origin firing: It is an essential cyclin-dependent kinase (CDK) substrate (4–6), its recruitment to origins is regulated by Dbf4-dependent kinase (DDK) (7, 8), it is inhibited by the DNA

damage checkpoint kinase Rad53 (9, 10), and it is one of a handful of limiting factors involved in executing the temporal program of origin firing (7, 11). Treslin/TICRR (TopBP1-interacting, replication stimulating protein/TopBP1-interacting, checkpoint, and replication regulator) is the ortholog of Sld3 in multicellular eukaryotes and is also regulated by CDK and the DNA damage checkpoint (12–16). Treslin/TICRR is, however, much larger than Sld3 and has additional domains, suggesting that it may interact with additional factors and may be regulated by additional pathways.

We used mass spectrometry to identify interacting proteins in FLAG immunoprecipitates from a

cell line stably expressing FLAG-tagged Treslin/TICRR. Among the proteins most highly enriched (table S1 and fig. S1) was the Mdm two binding protein (MTBP). Endogenous Treslin/TICRR and MTBP coimmunoprecipitated with antibodies to both MTBP and Treslin/TICRR (Fig. 1A). Depletion of MTBP with an antibody to MTBP resulted in removal of ~50% of the Treslin/TICRR from the extract (fig. S2i), and immunodepletion of Treslin/TICRR removed between 50 and 75% of the MTBP from the extract (fig. S2ii). MTBP and Treslin/TICRR coimmunoprecipitated from all extracts made from cells synchronized with either nocodazole or thymidine and released into the G₁, S, and G₂/M phases (Fig. 1B). In these experiments, MTBP and Treslin/TICRR were present throughout the cell cycle, but their levels increased later in the

cell cycle. Therefore, Treslin and MTBP are present in a complex throughout most or all of the cell cycle. MTBP interacted with Treslin/TICRR lacking the conserved CIT or STD domains (Fig. 2, A and B). However, two nonoverlapping deletions in the M domain between the CIT and STD domains (Δ M1 and Δ M2) showed reduced MTBP interaction, and one, Δ M2, was completely defective in MTBP interaction. Moreover, a fragment containing just the M-domain was sufficient for interaction with full-length MTBP (fig. S3). A small interfering RNA (siRNA)-resistant version of Δ M2 (Fig. 2C) localized to the nucleus (fig. S4) but could not restore DNA replication after depletion of endogenous Treslin/TICRR (Fig. 2D), indicating that Treslin/TICRR's ability to interact with MTBP is required for DNA replication.

Cancer Research UK London Research Institute (LRI), Clare Hall Laboratories, South Mimms, Herts. EN6 3LD, UK.
*Corresponding author. E-mail: john.diffley@cancer.org.uk

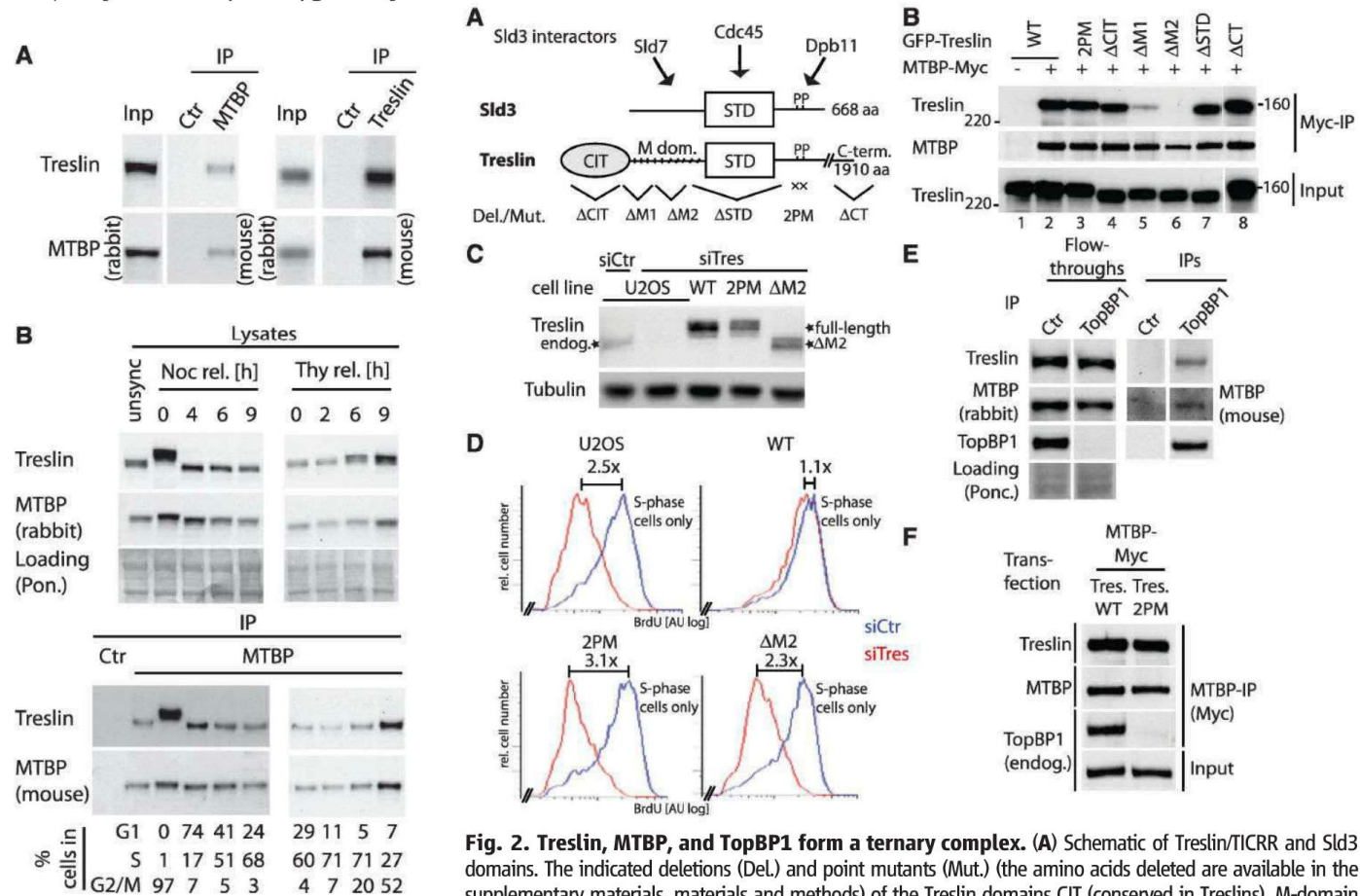


Fig. 1. MTBP and Treslin/TICRR form a complex throughout the cell cycle. (A) Endogenous MTBP or Treslin/TICRR were immunoprecipitated from HeLa cell extracts, and extracts (Inp) and immunoprecipitates (IP) were analyzed for MTBP and Treslin/TICRR with immunoblotting. Ctr, control-IPs. (B) U2OS cells were released from a mitotic block with nocodazole (Noc) or from a G₁/S arrest with thymidine (Thy) for the indicated times, and cell lysates and IPs of endogenous MTBP (MTBP-IP; Ctr-IP, control-IP) were analyzed with immunoblotting using antibodies to MTBP or Treslin/TICRR or Ponceau staining (Pon.). Cell-cycle analysis was done with flow cytometry of BrdU- and propidium iodide (PI)-stained cells.

Fig. 2. Treslin, MTBP, and TopBP1 form a ternary complex. (A) Schematic of Treslin/TICRR and Sld3 domains. The indicated deletions (Del.) and point mutants (Mut.) (the amino acids deleted are available in the supplementary materials, materials and methods) of the Treslin domains CIT (conserved in Treslins), M-domain (middle-domain), STD (Sld3/Treslin domain), TopBP1-interacting region [2PM, double phosphomutant (16)] and CT (C-terminus) were used in (B) to (D). (B) 293T cells were transiently transfected (16) with MTBP-Myc and AcGFP-Flag-Treslin-wild type (WT) or the indicated mutants described in (A). Myc-IPs and cell lysates (Input) were analyzed with immunoblotting with antibodies to Myc and GFP. 220/160 is the molecular weight in kilodaltons. (C) U2OS control cells or clones stably expressing siRNA-resistant AcGFP-Flag-Treslin-WT, 2PM, or Δ M2 (A) were analyzed by immunoblotting using antibodies to Treslin and Tubulin after control (siCtrl) or Treslin (siTres) RNAi (16). Asterisks indicate endogenous (endog.) and tagged Treslin/TICRR (Δ M2, full-length). (D) Cells described in (C) were analyzed for BrdU incorporation by using flow cytometry and displayed as histogram plots of FITC fluorescence (AU, arbitrary units; log, logarithmic scale). 2.5x, 1.1x, 3.1x, and 2.3x indicate fold decrease of FITC fluorescence upon Treslin-RNAi compared with siCtrl. (E) U2OS cell extracts were used for IPs with antibodies to TopBP1 or control (Ctr), and flow-throughs and IPs were analyzed by immunoblotting with antibody-to-Treslin, -MTBP, and -TopBP1 or with Ponceau staining (Pon.). (F) 293T cells were transiently transfected (16) with AcGFP-Flag-Treslin-WT or Treslin-2PM (16) and MTBP-Myc. Myc-IPs from native cell lysates (Input) were analyzed with immunoblotting using antibodies to GFP, Myc, and TopBP1.

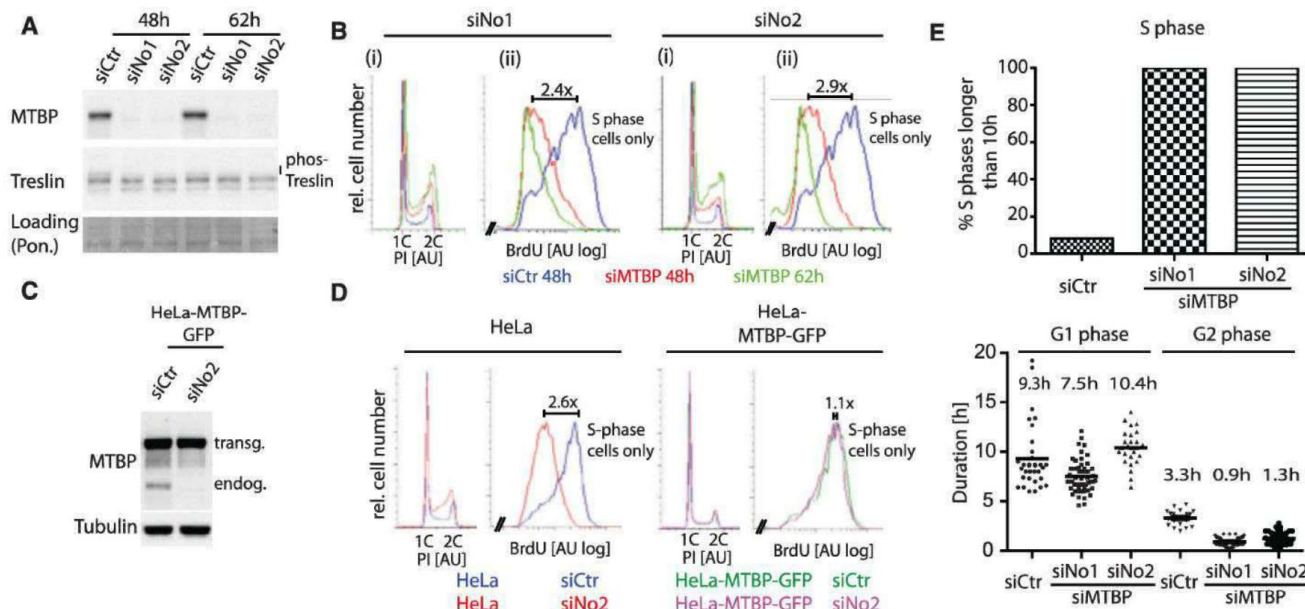


Fig. 3. Depletion of MTBP results in slower replication and longer S phases. (A) HeLa cells were treated with control (siCtrl) or MTBP (siNo1/2) siRNA for 48 or 62 hours, and whole cells were analyzed by immunoblotting with antibodies to MTBP and Treslin or with Ponceau (Pon.) staining. (B) Cells of (A) were analyzed for BrdU incorporation and chromatin content by PI (1C or 2C: G₁ or G₂/M phase) by using flow cytometry. (i) Whole-population PI histogram plots (AU, arbitrary units). (ii) Histograms of S phase cells stained with a FITC-coupled antibody to BrdU (log, logarithmic scale). 2.4x and 2.9x indicate fold decrease of FITC fluorescence upon MTBP-RNAi for 48 hours. (C) HeLa-MTBP-GFP

HeLa cells and clones stably expressing siRNA-resistant MTBP-Flag-AcGFP were depleted of endogenous MTBP for 48 hours as in (A). Immunoblots of whole-cell lysates were analyzed with antibodies to MTBP (endog., endogenous; transg., transgenic) and Tubulin. (D) The experiment for (C) was repeated and analyzed with flow cytometry as in (B). (E) Live-cell imaging of HeLa cells stably expressing GFP-PCNA was used to quantify G₁, S, and G₂ phases of the cell cycle upon treatment with control (siCtrl) or MTBP (siNo1/2) siRNAs. For technical reasons, S phase analysis is shown as the fraction longer than 10 hours, and G₁ and G₂ are in hours.

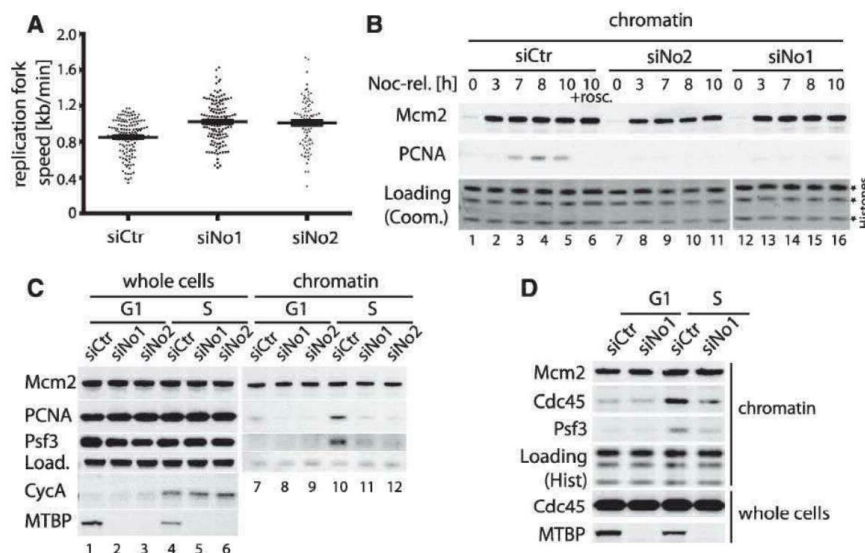


Fig. 4. MTBP is not required for replication elongation, licensing, and G₁-S transition but is required for GINS, Cdc45, and PCNA recruitment to chromatin in S phase. (A) MTBP was RNAi-depleted from HeLa cells as in Fig. 3A for 48 hours. Replication track lengths on chromatin fibers pulse-labeled with CldU were analyzed with fluorescence microscopy. Replication fork speeds are represented in kilobases per minute (kb/min) (error bars indicate SEM). (B) Control or MTBP-siRNA treated U2OS cells were released from a nocodazole block (Noc.-rel) for the indicated amounts of time. Chromatin-enriched fractions were analyzed for levels of Mcm2 and PCNA by means of immunoblotting and for histones by means of Coomassie (Coom.) staining. Rosc., roscovitine treatment from 3 hours after Noc.-release. (C) Control cells or cells depleted of MTBP were synchronized in the G₁ (3 hours nocodazole-release) or S phase (8 hours) as in (B), and chromatin or whole cells were analyzed by means of immunoblotting with antibodies against Mcm2, PCNA and Psf3, Cyclin A, MTBP, and Tubulin and by means of Ponceau staining. (D) Chromatin of cells treated as in (C) were analyzed by means of immunoblotting with a subset of antibodies used in (C) and with an antibody to Cdc45 and by means of Coomassie staining to detect histones (Hist).

MTBP interacted with the Δ CT mutant lacking the C-terminal tail and the 2PM mutant of Treslin/TICRR (Fig. 2B). The 2PM mutant lacks the two key CDK sites in the C terminus required for interaction of Treslin/TICRR with the multi-BRCT domain TopBP1 protein (15, 16). Therefore, the interaction of MTBP with Treslin/TICRR is not mediated by TopBP1. Endogenous MTBP coimmunoprecipitated with endogenous TopBP1 using antibodies to TopBP1 (Fig. 2E). However, TopBP1 was not coimmunoprecipitated with MTBP in cells transiently transfected with the 2PM mutant of Treslin/TICRR (Fig. 2F). Treslin/TICRR can thus interact simultaneously with TopBP1 and MTBP and bridges their interaction, which requires Treslin/TICRR phosphorylation by CDK.

MTBP depletion from HeLa cells with either of two siRNAs (siNo1 and siNo2) (Fig. 3A) reduced BrdU incorporation of cells in S phase without affecting Treslin/TICRR levels (Fig. 3A) and caused an accumulation of cells in late S phase (Fig. 3B, i, and fig. S6). These defects were suppressed by expression of an siRNA-resistant MTBP transgene fused at its C terminus to green fluorescent protein (GFP) (Fig. 3, C and D, and fig. S7), indicating that they are not off-target effects. An N-terminal GFP-MTBP fusion was unable to interact with Treslin/TICRR and did not suppress the replication phenotype of MTBP knockdown (fig. S8), providing additional evidence that the interaction between MTBP and Treslin/TICRR is critical for DNA replication.

Using time-lapse microscopy of cells expressing GFP–proliferating cell nuclear antigen (PCNA), we found that the S phase (distinguished by the presence of multiple nuclear PCNA foci) was >10 hours in virtually all cells in which either MTBP or Treslin/TICRR had been depleted (Fig. 3E and fig. S9) but was almost never this long in control treated cells, in which the S phase averaged 8.3 hours. The length of the G₁ phase was not affected in a consistent manner, but G₂ was greatly shortened. G₂ shortening may be a consequence of the greatly extended S phase or may reflect a second role for MTBP in G₂/M control (17). We conclude that depletion of MTBP inhibits DNA replication during the S phase. The small amount of replication seen after MTBP siRNA may indicate that some replication is MTBP-independent but is more likely due to incomplete MTBP depletion.

MTBP was discovered as a factor that interacted in a two-hybrid assay with MDM2 (18, 19), the E3 ubiquitin ligase involved in p53 degradation. We found, however, that MTBP depletion inhibited replication in both p53-deficient HeLa cells (Fig. 3) and p53-positive U2OS cells (fig. S6), as well as p53-positive and -negative HCT116 cells (fig. S10A). In addition, inhibition of the MDM2 ubiquitin ligase with nutlin-3 did not alleviate the inhibition of DNA replication in p53-deficient cells upon MTBP RNA interference (RNAi) (fig. S10, B and C). These results indicate that MTBP is essential for DNA replication regardless of a cell's p53 status and are consistent with previous work showing that MTBP is essential for early mouse development in both p53^{+/+} and p53^{-/-} backgrounds (20). Although p53 was activated upon MTBP depletion (fig. S11) as previously reported (19), this happened at relatively late times and is likely to be a consequence of aberrant replication because cells accumulate in S and G₂ phases rather than the G₁ phase (Fig. 3B and figs. S5 and S6). Although p53 is not required for MTBP's function in replication, it is intriguing to consider that MTBP might play a role in regulating p53, perhaps coupling some aspect of origin surveillance with G₁/S progression.

We next used DNA fiber labeling (21, 22) to examine rates of DNA replication fork progression. Although MTBP knockdown reduced overall DNA synthesis (Fig. 3B), the lengths of remaining nascent DNA synthesis tracks were not shorter after MTBP knockdown (Fig. 4A), indicating that the defect in replication is likely due to reduced origin firing rather than reduced fork rate. Mcm2 bound to chromatin normally as cells progressed through mitosis and into G₁ phase (3 hours after nocodazole release) after MTBP knockdown (Fig. 4B), indicating that MTBP is not required for mitotic progression or origin licensing. However, considerably less PCNA was associated with chromatin at later times (for example, 7 to 10 hours after release) when MTBP was depleted, similar to treatment with the CDK inhibitor roscovitine (lane 6). Levels of GINS (Fig. 4C) and Cdc45 (Fig. 4D), components of the CMG helicase, were also reduced on chromatin after MTBP knockdown. The decrease in PCNA, Cdc45, and GINS

chromatin association is not due to a failure of progression into S phase because the E2F-induced cyclin A was expressed to the same level as in control cells (lanes 4 to 6). Therefore, MTBP is required after origin licensing for CMG assembly.

MTBP may have a similar role to yeast Sld7 (23), given that both proteins interact with analogous regions of Treslin/TICRR and Sld3 and that a homolog of Sld7 has not been identified in humans. MTBP is amplified in >10% breast cancers and >20% ovarian cancers as part of a large amplicon on chromosome 8q24 that includes the MYC gene (24, 25). Mouse models have shown that MTBP haploinsufficiency significantly decreases myc-induced lymphomagenesis (26), suggesting that this step in DNA replication may be a useful drug target for some cancers.

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Supplementary Materials

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The Human Malaria Parasite *Pfs47* Gene Mediates Evasion of the Mosquito Immune System

Alvaro Molina-Cruz,¹ Lindsey S. Garver,¹ Amy Alabaster,¹ Lois Bangiolo,¹ Ashley Haile,¹ Jared Winikor,¹ Corrie Ortega,¹ Ben C. L. van Schaijk,² Robert W. Sauerwein,² Emma Taylor-Salmon,¹ Carolina Barillas-Mury^{1*}

Plasmodium falciparum transmission by *Anopheles gambiae* mosquitoes is remarkably efficient, resulting in a very high prevalence of human malaria infection in sub-Saharan Africa. A combination of genetic mapping, linkage group selection, and functional genomics was used to identify *Pfs47* as a *P. falciparum* gene that allows the parasite to infect *A. gambiae* without activating the mosquito immune system. Disruption of *Pfs47* greatly reduced parasite survival in the mosquito, and this phenotype could be reverted by genetic complementation of the parasite or by disruption of the mosquito complement-like system. *Pfs47* suppresses midgut nitration responses that are critical to activate the complement-like system. We provide direct experimental evidence that immune evasion mediated by *Pfs47* is critical for efficient human malaria transmission by *A. gambiae*.

The mosquito *Anopheles gambiae* is the natural vector of the human malaria parasite *Plasmodium falciparum* in large regions of Africa. There is compelling evidence, mostly from murine models, that *A. gambiae* can mount robust and effective antiparasitic responses. For example, midgut epithelial cells activate nitration reactions in response to *Plasmodium* in-

fection that limit parasite survival (1, 2) by modifying the parasite and promoting lysis by thioester-containing protein 1 (TEP1), a key component of the mosquito complement-like system (3). However, several studies indicate that disrupting the complement-like system has a modest (4, 5) or no (6) effect when *A. gambiae* is infected with *P. falciparum*.

The *A. gambiae* L3-5 strain was selected to be refractory (R) to *Plasmodium cynomolgi* (simian malaria), but it also eliminates most other *Plasmodium* species, including *P. falciparum* strains from the New World, and forms a melanotic capsule (i.e., deposition of melanin, a black insoluble pigment) around the dead parasites. In contrast, this strain is highly susceptible to infection with some African *P. falciparum* strains, such as NF54, 3D7, and GB4 (7, 8). Some parasite lines from

malaria-endemic areas where *A. gambiae* is the natural vector are able to evade the mosquito immune system (8). Coinfection experiments reveal that the immune response (or lack thereof) to a *P. falciparum* strain did not affect the fate of other parasites present in the same mosquito midgut (8), suggesting that parasite survival is determined by genetic differences between *P. falciparum* strains (8). In this study, quantitative trait locus (QTL) mapping, linkage group selection, and functional genomics were used to identify the first *P. falciparum* gene that promotes infection by modulating the host immune system.

We took advantage of the phenotypic difference between *A. gambiae* R infected with two *P. falciparum* lines—7G8 from Brazil (97 to

100% melanized) and GB4 from Ghana (0 to 3% melanized) (8) (Fig. 1A)—that have been previously subjected to a genetic cross (9). We phenotyped nine cloned progeny lines, five of them had the GB4 phenotype and survived well (0 to 5% melanization), whereas four had the 7G8 phenotype and were mostly melanized (98 to 100% melanization) (Fig. 1B) (10). The presence of two distinct phenotypes in the progeny suggested a monogenic trait. Repeated attempts to phenotype 16 additional progeny lines failed because most clonal lines had lost the ability to generate mature gametocytes. QTL mapping, using a previously reported linkage map (9) and the phenotypes obtained, identified three logarithm of odds (LOD) peaks (Fig. 1C), but only one of them, located in

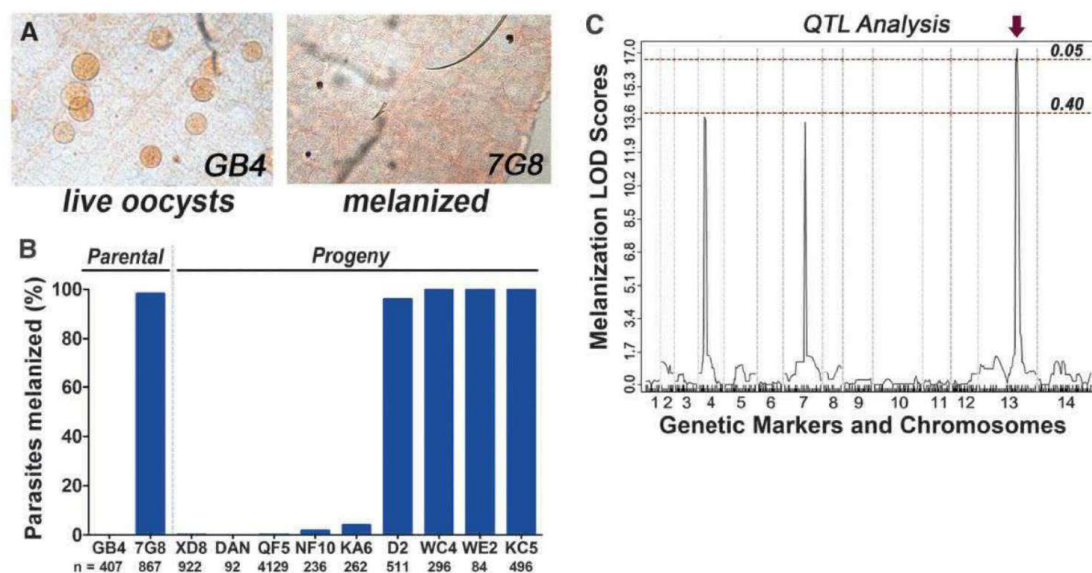


Fig. 1. Survival of the parental and progeny *P. falciparum* lines in R mosquitoes and QTL mapping of the melanization phenotype. (A) *P. falciparum* GB4 and 7G8 parasites in the midgut of R mosquitoes. (B) Melanization phenotype of the parental and progeny lines of the GB4 × 7G8 genetic cross in R mosquitoes. (C) LOD scores of genome-wide QTL analysis of the melanization phenotype. Red dotted lines, statistical significance thresholds at $P = 0.05$ and $P = 0.40$; arrow, significant QTL.

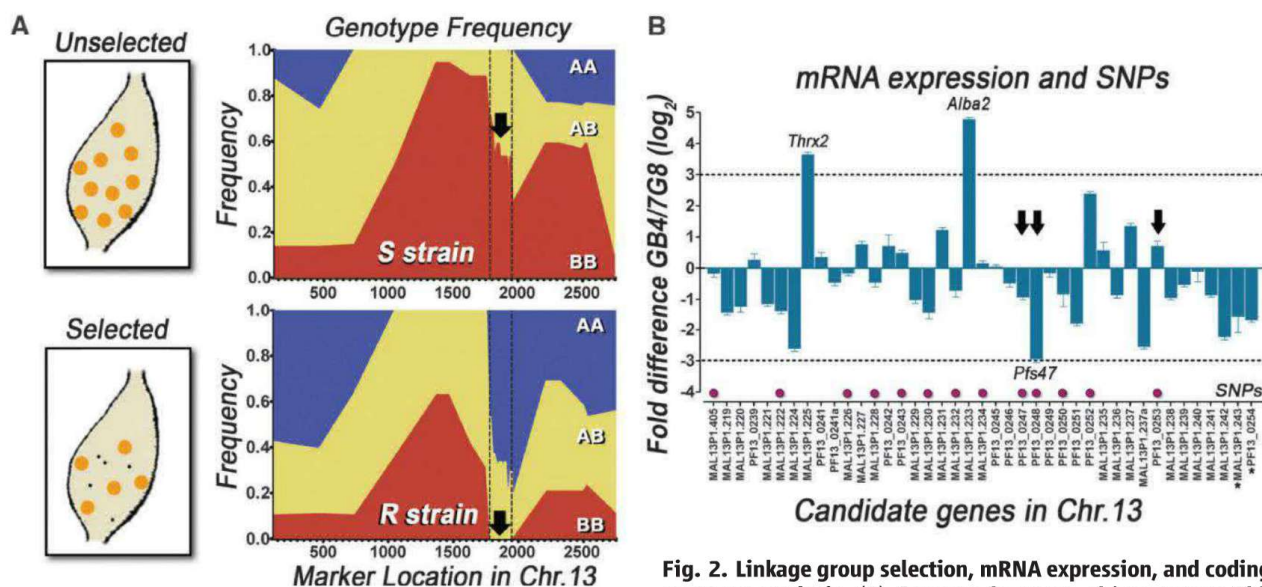


Fig. 2. Linkage group selection, mRNA expression, and coding region sequence analysis. (A) Genotype frequency of homozygous African (AA, blue) or Brazilian (BB, red) or heterozygous (AB, yellow) markers along Chr13 in individual oocysts dissected from S or R mosquitoes. Black arrow, region with BB under extreme negative selection in R strain. (B) Relative mRNA expression of candidate genes between GB4 and 7G8 *P. falciparum* ookinete stage. Magenta dots, genes with nonsynonymous SNPs between GB4 and 7G8; arrows, SNPs shared between GB4-3D7 and 7G8-SL strains.

in individual oocysts dissected from S or R mosquitoes. Black arrow, region with BB under extreme negative selection in R strain. (B) Relative mRNA expression of candidate genes between GB4 and 7G8 *P. falciparum* ookinete stage. Magenta dots, genes with nonsynonymous SNPs between GB4 and 7G8; arrows, SNPs shared between GB4-3D7 and 7G8-SL strains.

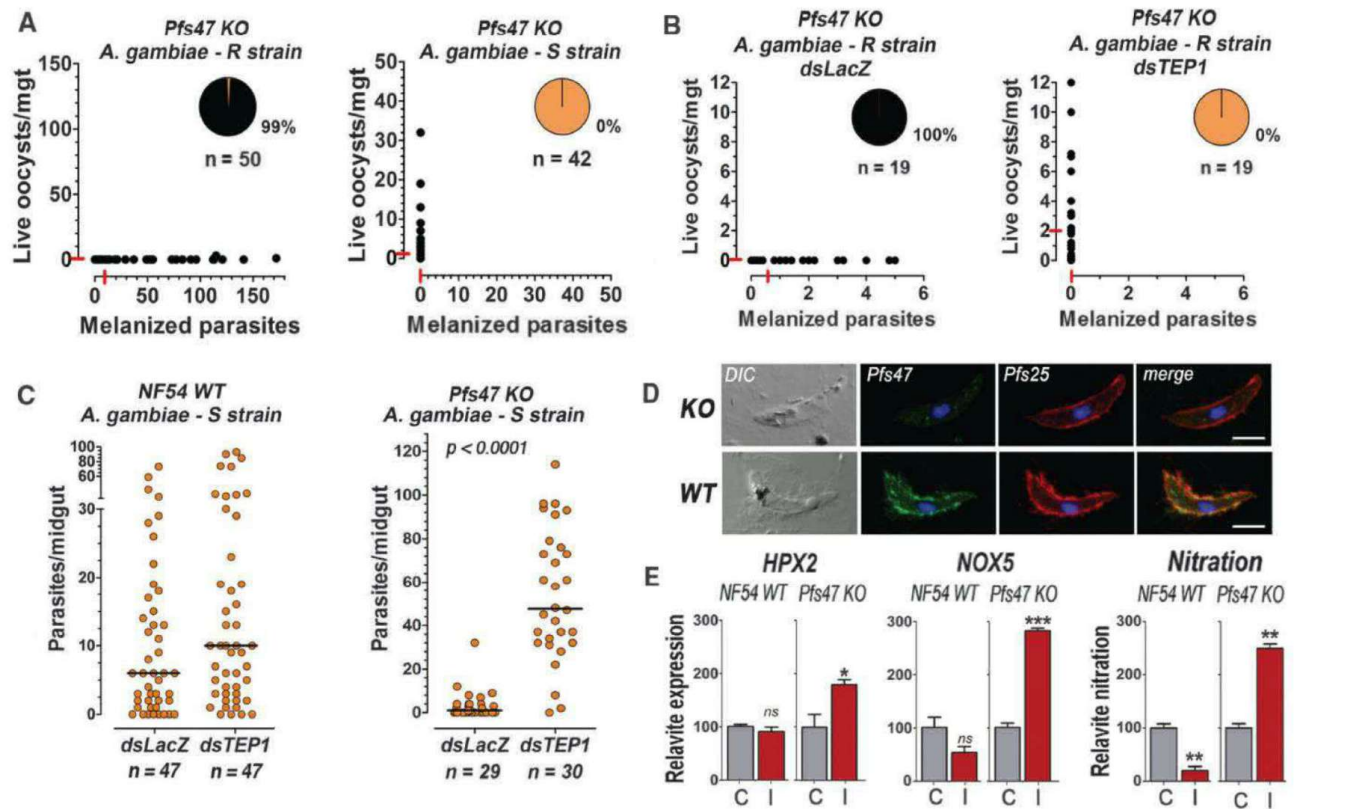
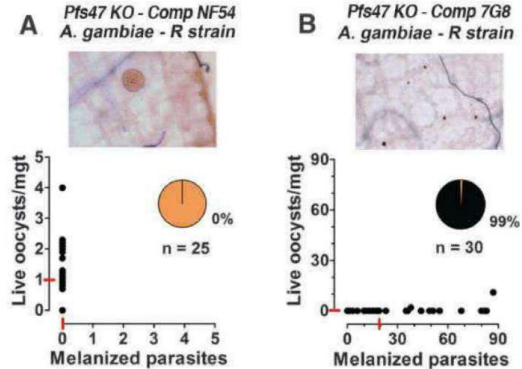


Fig. 3. Phenotype of *NF54* WT or *Pfs47* KO on different mosquitoes. (A) Number of melanized (x axis) and live (y axis) KO parasites in R and S mosquitoes. Each dot represents an individual midgut. Medians are indicated by the red lines. (B) Effect of TEP1 silencing on KO infection in R mosquitoes. (C) Effect of silencing TEP1 on WT and KO infection in S mosquitoes. (D)

Immunofluorescence staining of WT and KO ookinetes with *Pfs47* (green) and *Pfs25* (red) (scale bar, 5 μ m). (E) Midgut mRNA expression of *HPX2* and *NOX5*, and midgut nitration using enzyme-linked immunosorbent assay, 24 hours after S females were infected with WT or KO parasites (I, infected) or fed uninfected blood (C, control).

chromosome 13 (Chr13), was significant ($P < 0.05$). The boundaries of the recombination sites for this locus were precisely mapped and defined a 172-kb region coding for 41 genes (fig. S1 and table S1). Linkage group selection analysis, a method that allows de novo location of loci encoding selectable phenotypes of malaria parasites (11), was used to obtain independent confirmation of the locus in Chr13. The uncloned recombinant progeny from the original genetic cross was used to generate gametocytes and infect either the R strain or a permissive susceptible (S) *A. gambiae* G3 line in which both parental parasite lines survive. Individual oocysts were isolated, subjected to whole-genome DNA amplification, and genotyped for multiple markers along Chr13. Oocysts derive from the diploid ookinete stage and can be homozygous for the African GB4 (AA) or Brazilian 7G8 alleles (BB) or heterozygous (AB). In the S strain, the BB genotype is highly abundant in the central region of Chr13, reaching a frequency of $>90\%$ (Fig. 2A, fig. S2, and table S2), which was already observed in the progeny clones from the genetic cross (9) and is not due to selection by the mosquito, because both parental strains survive in the S strain (7). In the R strain, we identified a well-defined region, indicated by the dotted line, in which the BB genotype is under strong negative selection and is totally absent (0%) (Fig. 2A). In

Fig. 4. Effect of complementing *Pfs47* KO parasites with the Brazilian (7G8) and African (*NF54*) alleles of *Pfs47*. Infectivity of *Pfs47* KO parasites complemented with the (A) *NF54* or (B) 7G8 *Pfs47* alleles in the *A. gambiae* R strain.



contrast, prevalence of the BB genotype in the same chromosomal region is 55% in the S strain ($P < 0.00001$; χ^2 test), which does not exert selective pressure on the parasite. It is noteworthy that the two markers that define this region (Fig. 2A, dotted lines) are the same as those that limit the 172-kb region identified by QTL analysis. Although 50 additional individual oocysts dissected from the R strain were genotyped, no oocyst with the BB genotype was detected for any of the markers within the region under strong selection (fig. S3). The locus could therefore not be narrowed down any further. Gene expression analysis of the 41 candidate genes in the ookinete stage identified three genes

with large differences in expression (eightfold or higher) between the parental lines: *Pfs47* (PF13_0248), *thioredoxin 2* (MAL13P1.225), and the nucleic acid binding protein *ALBA2* (MAL13P1.233) (Fig. 2B and table S3) ($P < 0.0001$; t test). *Thioredoxin 2* and *ALBA2* also had differences in expression between the parental lines in the gametocyte stage (table S3). Sequencing the coding regions of the 41 candidate genes identified nonsynonymous single nucleotide polymorphisms (SNPs) between the parental lines in 13 genes (Fig. 2B, magenta dots and table S4). Some nonsynonymous SNPs in three of these genes—four SNPs in *Pfs47* and one in *Pf48/45* (PF13_0247) and in *ethanolamine-*

phosphate cytidyltransferase (PF13_0253)—also correlate with parasite survival in two other *P. falciparum* strains (Fig. 2B, black arrows, and table S4). The GB4 SNP alleles are shared with the NF54 and 3D7 strains that also survive, and the 7G8 SNP alleles are shared with the SL strain that is melanized by the R strain (7). Five genes were selected as top candidates for detailed genetic analysis on the basis of large differences in gene expression and/or on polymorphisms that correlates with survival in other strains (table S5).

Two top candidate genes, *Pfs47* and *Pfs48/45*, code for members of the 6-cysteine protein family that are expressed on the gametocyte surface. Previous gene disruption experiments in the NF54 line revealed that *Pfs48/45* is critical for gamete fertility (12). *Pfs47* is expressed in female gametocytes but is not essential for *P. falciparum* fertilization, although its homolog in *Plasmodium berghei* is required for female gamete fertility (12, 13). The intensity of infection with the *Pfs48/45* knockout (KO) line (NF54 genetic background) in the R strain was low, probably because of reduced fertility. However, those parasites that invaded the midgut had a similar phenotype as wild-type (WT) NF54 parasites (8), and only 3% were melanized (fig. S4), indicating that *Pfs48/45* is not required to evade the mosquito immune system. In contrast, *Pfs47* KO (NF54 genetic background) parasites develop and invade the midgut but are eliminated by R mosquitoes (99% melanization) (Fig. 3A); although *Pfs47* KO parasites are not melanized by S mosquitoes (Fig. 3A), the infection level in the S strain (median of one oocyst per midgut) is much lower than that in *A. stephensi* mosquitoes (60 oocysts per midgut median) (fig. S5). This *A. stephensi* strain has been selected to be highly permissive to *P. falciparum* infection (14).

To determine whether *Pfs47* interacts with the mosquito immune system, we disrupted the *A. gambiae* complement-like system by silencing TEPI. Reducing TEPI expression completely reversed melanization of *Pfs47* KO parasites in the R strain (Fig. 3B). In the *A. gambiae* S strain (G3), neither NF54 WT nor *Pfs47* KO parasites were melanized (Fig. 3C); although TEPI silencing had no significant effect on infection with NF54 WT parasites (Fig. 3C), it increased both the intensity ($P < 0.0001$, Mann-Whitney test) and the prevalence of infection ($P < 0.001$; χ^2 test) of *Pfs47* KO parasites (Fig. 3C). This result indicates that *Pfs47* is necessary for *P. falciparum* parasites to evade two well-characterized immune responses mediated by TEPI in *A. gambiae*: killing followed by melanization in the R strain and parasite lysis without melanization in the S strain.

Pfs47 protein is present on the surface of WT NF54 ookinetes, the stage that invades the midgut, but is absent in *Pfs47* KO parasites (Fig. 3D). The expression of HPX2 and NOX5, two enzymes that mediate midgut nitration in response to *P. berghei* infection and promote TEPI activation (2), was evaluated in S mosquitoes. HPX2 and NOX5 were not induced by NF54 WT parasites, and nitration

levels were lower than in uninfected controls (Fig. 3E). In contrast, *Pfs47* KO parasites induced expression of HPX2 and NOX5 and a robust nitration response, indicating that *Pfs47* may prevent TEPI-mediated lysis by suppressing midgut epithelial nitration responses (Fig. 3E).

Finally, we confirmed the importance of *Pfs47* for parasite survival by complementing the *Pfs47* KO line with different *Pfs47* alleles (figs. S6 to S9). As expected, the NF54 allele of *Pfs47* reversed the melanization phenotype (0% melanization) in the R strain when the complemented parasites were kept under sustained drug pressure, confirming that this allele of *Pfs47* is sufficient to evade the immune system (Fig. 4A). A reversal to a mixed live/melanization phenotype was observed when the drug pressure was reduced (fig. S10). In contrast, complementation with the 7G8 allele failed to rescue parasites in the R strain, because 99% melanization was observed (Fig. 4B).

Together, our findings identify *Pfs47* as an essential survival factor for *P. falciparum* that allows the parasite to evade the immune system of *A. gambiae*, a major mosquito vector in Africa. However, other parasite genes may also be involved in this process. *Pfs47* is a highly polymorphic gene with a marked population structure in field isolates and exhibits extreme fixation in non-African regions of the world (15, 16). Our findings suggest that the population structure of *Pfs47* may be due to adaptation of *P. falciparum* to the different *Anopheles* vector species present outside of Africa. The fact that the 7G8 allele of *Pfs47* is sufficient to evade the TEPI complement-like system in S mosquitoes but not in the R strain indicates that there are also genetic differences in the vector that determine compatibility with parasites that express specific *Pfs47* alleles. It appears that *Pfs47* evolved a function in *P. falciparum* that increases parasite survival in *A. gambiae* mosquitoes and may be responsible, at least in part, for the very high rates of malaria

transmission in hyperendemic regions in Africa. Disruption of the immunomodulatory activity of *Pfs47* may prove to be an effective strategy to reduce malaria transmission to humans.

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Supplementary Materials

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Figs. S1 to S9

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Tetrahydrobiopterin Biosynthesis as an Off-Target of Sulfa Drugs

Hirohito Haruki,¹ Miriam Grønlund Pedersen,¹ Katarzyna Irena Gorska,¹ Florence Pojer,² Kai Johnsson^{1*}

The introduction of sulfa drugs for the chemotherapy of bacterial infections in 1935 revolutionized medicine. Although their mechanism of action is understood, the molecular bases for most of their side effects remain obscure. Here, we report that sulfamethoxazole and other sulfa drugs interfere with tetrahydrobiopterin biosynthesis through inhibition of sepiapterin reductase. Crystal structures of sepiapterin reductase with bound sulfa drugs reveal how structurally diverse sulfa drugs achieve specific inhibition of the enzyme. The effect of sulfa drugs on tetrahydrobiopterin-dependent neurotransmitter biosynthesis in cell-based assays provides a rationale for some of their central nervous system–related side effects, particularly in high-dose sulfamethoxazole therapy of *Pneumocystis pneumonia*. Our findings reveal an unexpected aspect of the pharmacology of sulfa drugs and might translate into their improved medical use.

Tetrahydrobiopterin (BH₄) is a cofactor of aromatic hydroxylases, such as phenylalanine, tyrosine and tryptophan hydroxylases, all isoforms of nitric oxide synthases, and alkylglycerol monooxygenase (1). BH₄ deficiencies result in symptoms of hyperphenyl-

ylases, all isoforms of nitric oxide synthases, and alkylglycerol monooxygenase (1). BH₄ deficiencies result in symptoms of hyperphenyl-

alaninemia, as well as dopamine and serotonin (5-hydroxytryptamine) deficiencies (2, 3). The final step in BH₄ biosynthesis is the reduction of 6-pyruvoyltetrahydropterin by sepiapterin reductase (SPR) (Fig. 1A). BH₄ is also recycled from the oxidized form of the cofactor and synthesized by a salvage pathway via dihydrobiopterin (BH₂) (Fig. 1A). Recently, we showed that the anti-inflammatory drug sulfasalazine and its metabolite sulfapyridine are potent inhibitors of SPR and proposed that an inhibition of BH₄ biosynthesis by the highly permeable sulfapyridine is responsible for the anti-inflammatory activity of sulfasalazine in rheumatoid arthritis (4). Here, we report that the inhibition of BH₄ biosynthesis contributes to the pharmacological properties of other clinically approved drugs. By screening a library of approved drugs for SPR inhibitors, we identified 10 drugs that inhibited SPR with median inhibitory concentration (IC₅₀) values below 100 nM (Fig. 1B and tables S1 and S2). These included the known SPR inhibitors sulfasalazine and sulfapyridine. Furthermore, the antibacterial sulfa drugs—sulfathiazole, sulfamethoxazole, sulfamethizole, phtalylsulfathiazole, and sulfadiazine, as well as the three antidiabetic sulfonylureas chlorpropamide, glibenclamide, and tolbutamide—were identified as potent SPR inhibitors. To gain an understanding of the binding mode of the different sulfonamides to SPR, we solved the crystal structures of SPR with bound nicotinamide adenine dinucleotide phosphate (NADP⁺) and sulfapyridine or sulfathiazole. SPR, NADP⁺, and sulfapyridine or sulfathiazole form a ternary complex in which the sulfa drugs block the substrate binding site (Fig. 2, A and B). The two drugs bind to SPR in a near-identical fashion (Fig. 2B), and there are no substantial differences detectable between the two protein structures (root-mean-square deviation between backbone Cα atoms of 0.1 Å). The sulfonamide, the moiety common to all hits, and the nitrogen atom in the heteroaromatic ring of sulfapyridine and sulfathiazole form specific hydrogen bonds with active site residues Ser¹⁵⁷, Tyr¹⁷⁰, and Asp²⁵⁷ (Fig. 2C). A hydrogen bond acceptor at a position equivalent to that of the nitrogen atom in the pyridine ring of sulfapyridine is found in all identified SPR inhibitors (Fig. 1B); in the sulfonylureas, the oxygen atom of the urea group could function as a hydrogen bond acceptor. Previous structural studies on mouse SPR have revealed that these three residues are important for substrate binding (5). The pyridine or thiazole rings also stack on top of the nicotinamide ring of NADP⁺ (Fig. 2B) and make hydrophobic contacts with residues Leu¹⁰⁴, Trp¹⁶⁷, Met²⁰⁵, and Ala²⁰⁹. The different heterocycles or alkyl groups

found in other SPR inhibitors (Fig. 1B) should be able to bind in an equivalent fashion as the heteroaromatic ring of sulfapyridine and sulfathiazole. Our structural data thus suggest how structurally diverse sulfonamides achieve inhi-

bition of SPR but also why certain sulfa drugs, such as sulfamethazine (Fig. 1B), are only very weak inhibitors of SPR. Sulfamethazine is a weaker inhibitor of SPR than sulfadiazine, which is 370 times as strong, although it differs from

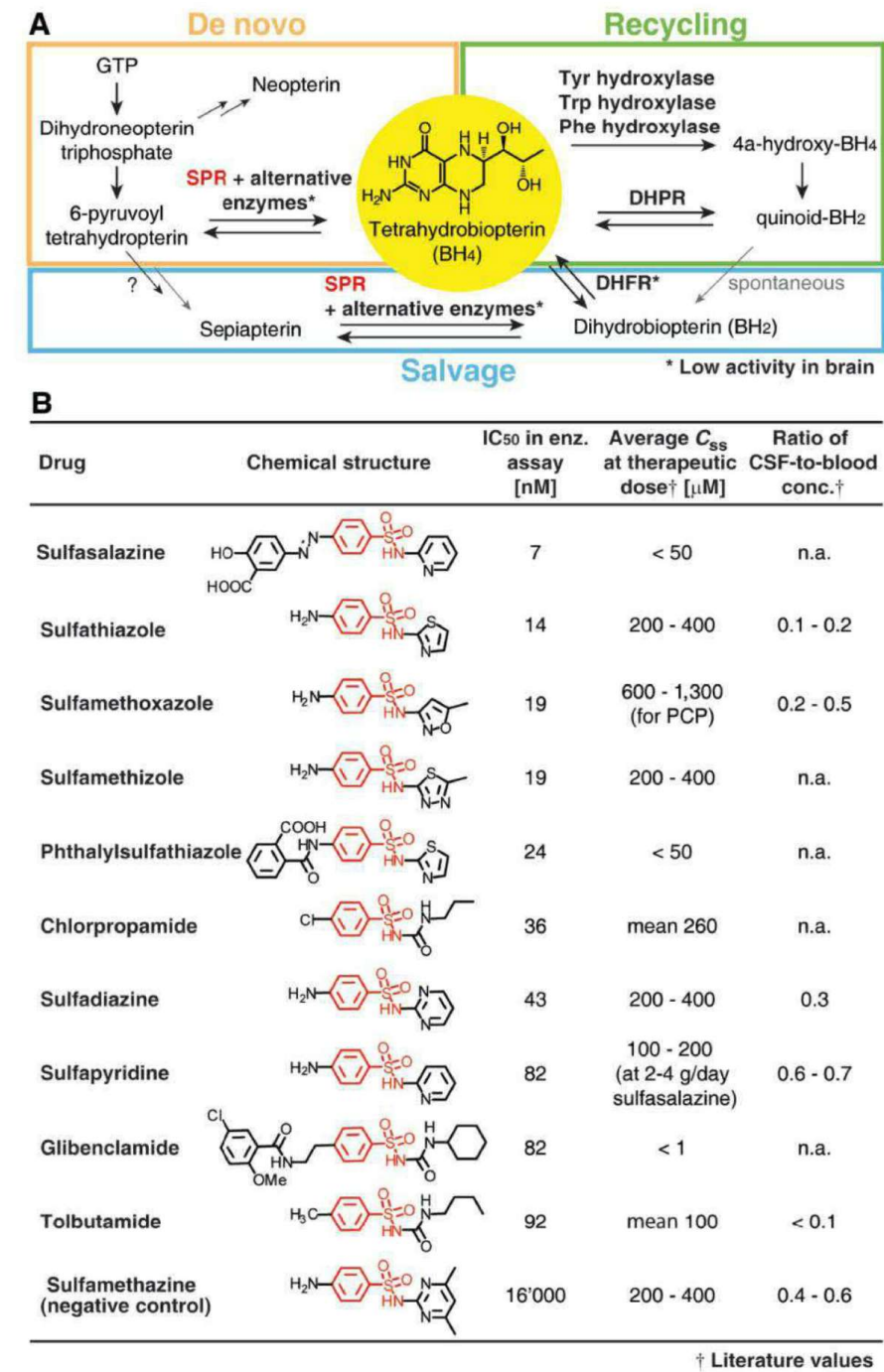


Fig. 1. Pathways for BH₄ homeostasis and structures of SPR inhibitors. (A) BH₄ biosynthetic pathways. SPR catalyzes formation of BH₄ from 6-pyruvoyltetrahydropterin and of BH₂ from sepiapterin in a salvage pathway. (B) SPR inhibitors, their IC₅₀ values for inhibition of SPR in an enzymatic assay, and some of their pharmacokinetic properties. IC₅₀ values are means of triplicate experiments (N = 2 at least) (full data in table S2). The benzenesulfonamide moiety found in all SPR inhibitors is highlighted in red. Average steady-state serum or blood concentrations (C_{ss}) at therapeutic dose and the ratio of CSF-to-blood concentrations are literature values (see references in table S3). The listed ratio of CSF-to-blood concentration of tolbutamide was determined in rats (26, 27). Sulfamethazine, which only weakly inhibits SPR, served as negative control throughout this work. n.a., not available.

¹École Polytechnique Fédérale de Lausanne (EPFL), Institute of Chemical Sciences and Engineering, Institute of Bioengineering, National Centre of Competence in Research (NCCR) in Chemical Biology, 1015 Lausanne, Switzerland. ²EPFL, Global Health Institute, 1015 Lausanne, Switzerland.

*Corresponding author. E-mail: kai.johnsson@epfl.ch

sulfadiazine only by two additional methyl groups attached to the pyrimidine ring (Fig. 1B). A manual superimposition of sulfamethazine on sulfapyridine in the structure of SPR reveals an

energetically unfavorable interaction of the two additional methyl groups with the side chains of Met²⁰⁵ and Gln²⁰⁶, respectively; the distance of the sulfur atom of Met²⁰⁵ and the amide ni-

trogen of Gln²⁰⁶ to the nearest methyl group is 2.55 Å and 2.75 Å, respectively (fig. S1, D and E). These distances are more than 0.5 Å below the corresponding nonbonded contact distances observed in organic crystals and protein structures (6, 7).

Next, we examined if these drugs reduce BH₄ levels in human primary dermal fibroblasts in which BH₄ biosynthesis is induced by pro-inflammatory cytokines. Incubation of human fibroblasts with interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF α) for 24 hours increased BH₄ levels 165-fold to 0.33 nmol/mg protein (Fig. 3A). SPR inhibitors decreased BH₄ levels in cytokine-stimulated fibroblasts in a dose-dependent manner, whereas sulfamethazine had no effect on BH₄ levels. Dihydroneopterin triphosphate (DHNTTP) is a precursor of the SPR substrate 6-pyruvoyltetrahydropterin in the biosynthesis of BH₄ (Fig. 1A), and increased DHNTTP levels have been detected in cytokine-stimulated fibroblasts derived from SPR-deficient patients (8). Accordingly, treatment with SPR inhibitors significantly increased DHNTTP levels (measured as neopterin after phosphatase treatment) (Fig. 3A and Fig. 1A), which confirmed that the observed decrease in BH₄ is due to an inhibition of SPR. Note that, the observed inhibition of BH₄ biosynthesis occurs at drug concentrations that are within their reported serum levels (Fig. 1B). The exception in these experiments was sulfasalazine, which decreased BH₄ levels only at high micromolar concentrations and mainly by inhibition of cytokine signaling (fig. S2). The low potency of sulfasalazine as SPR inhibitor in cell-based assays can be attributed to its low cell permeability (9).

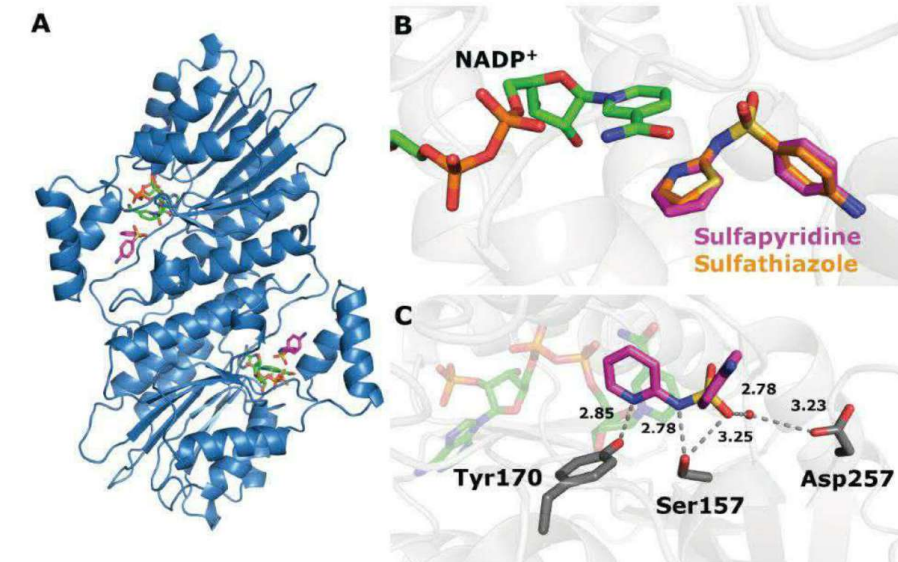
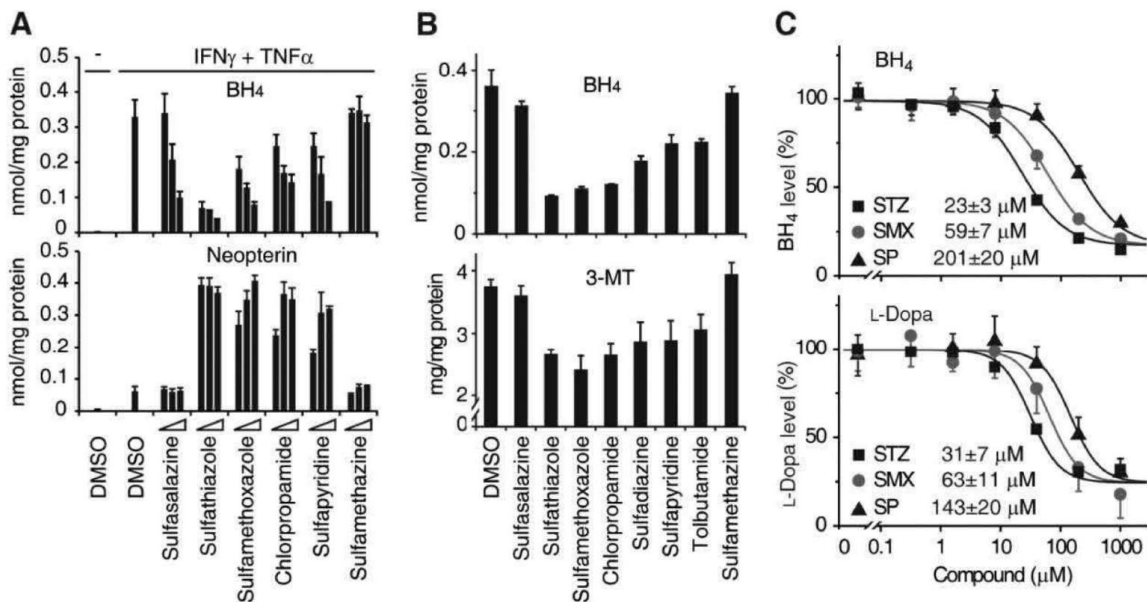


Fig. 2. Binding mode of sulfa drugs to SPR. (A) Overview of crystal structure of human SPR in complex with NADP⁺ (green) and sulfapyridine (magenta). (B) Close-up of a 2.4 Å crystal structure of human SPR with bound NADP⁺ and sulfapyridine. In the ternary complex, the pyridine ring of sulfapyridine is stacked on top of the nicotinamide ring of NADP⁺ with a distance of 3.3 Å between the nitrogen in the pyridine ring and C4 of the nicotinamide ring. The overlay of sulfapyridine with sulfathiazole (orange) from a 2.45 Å crystal structure of human SPR with bound NADP⁺ and sulfathiazole shows that the two drugs bind to SPR in a near-identical fashion. (C) Key hydrogen bonds between the sulfonamide moiety of sulfapyridine and active site residues Ser¹⁵⁷, Tyr¹⁷⁰, and Asp²⁵⁷ known to be important for substrate binding (5). A water molecule (red) mediates the hydrogen bond between Asp²⁵⁷ and sulfapyridine. Distances are given in Å.

Fig. 3. Inhibition of BH₄ and L-dopa synthesis by sulfonamides.

(A) Effect of selected drugs on BH₄ synthesis in cytokine-stimulated human primary dermal fibroblasts. Intracellular BH₄ and neopterin levels were measured after 24 hours concomitant treatment of IFN- γ and TNF α and SPR inhibitors (0.01, 0.1, and 1 mM). Data are means $\pm$ SD from triplicate experiments [$N = 1$, except $N = 3$ for dimethyl sulfoxide (DMSO) + cytokines]. (B) Effect of selected drugs on BH₄ and extracellular 3-MT levels in SK-N-BE(2) cells. Cells were incubated with drugs for 24 hours at 200 μ M concentrations. Intracellular BH₄ level and 3-MT level in culture supernatant were measured. Data are means $\pm$ SD from triplicate experiments ($N = 1$). (C) Effect of sulfamethoxazole (SMX), sulfathiazole (STZ), and sulfapyridine (SP) on BH₄ synthesis and L-dopa levels in SK-N-BE(2) cells. Intracellular BH₄ levels were measured after 24-hour



treatment of cells with drugs. Extracellular L-dopa levels were measured after 24-hour treatment of cells with drugs, followed by 30 min of treatment with 10 μ M NSD-1015, aromatic L-amino acid decarboxylase inhibitor. Data are means $\pm$ SD from triplicate experiments ($N = 1, 2, 3$ for STZ, SP, and SMX, respectively).

Sulfamethoxazole is currently the most-used sulfa drug and is prescribed in combination with trimethoprim (cotrimoxazole) for microbial infections and as standard treatment against *Pneumocystis pneumonia* (PCP), a prevalent opportunistic fungal infection in immunocompromised individuals (10, 11). Both sulfamethoxazole and trimethoprim target pathogen tetrahydrofolate synthesis; sulfamethoxazole is a competitive inhibitor of dihydropteroate synthase, whereas trimethoprim inhibits dihydrofolate reductase (DHFR). Adverse effects of sulfamethoxazole-trimethoprim at standard dose for bacterial infection are relatively rare, but CNS-related side effects are frequently observed at the higher therapeutic dose used in PCP (up to 100 mg/kg of body weight per day sulfamethoxazole and 20 mg/kg per day of trimethoprim). These include anorexia, nausea, headache, nervousness, fine tremors, lightheadedness, insomnia, and drowsiness (see table S4 for a compilation of reports concerning CNS-related adverse effects). For example, administering 100 mg/kg per day of sulfamethoxazole to 12 healthy adults resulted in CNS-related adverse effects in all subjects within 3 days (12). Furthermore, recent analyses of clinical data of PCP patients indicate that high-dose sulfamethoxazole-trimethoprim therapy can cause psychosis; the reported incidence being 12 to 20% (13, 14). Nausea, vomiting, and CNS-related symptoms are common, dose-dependent adverse effects of most sulfa drugs (15–17). In sulfasalazine treatment, these symptoms are correlated with serum concentrations of sulfapyridine but not sulfasalazine (18). In dogs, which exhibit CNS-related side effects similar to those of humans (19), complete gastrointestinal eviceration or direct application of sulfapyridine to the vomiting center showed that nausea and vomiting are not caused by direct action of the drug on the gastrointestinal tract or the vomiting center (20). The inhibition of BH₄ biosynthesis by sulfa drugs and a subsequent inhibition of neurotransmitter biosynthesis could provide a rationale for their CNS-related side effects. This hypothesis is supported by the observation that the adverse effects of sulfamethoxazole resemble the symptoms of genetically inherited SPR deficiency. Basal levels of BH₄ in SPR deficiency in most tissues are maintained through salvage or alternative pathways (Fig. 1A). An exception is the brain, where the low expression level of the enzymes involved in salvage pathways, such as DHFR, diminishes their efficiency. Symptoms of SPR deficiency are those of catecholamine and serotonin deficiencies with disabling motor and cognitive deficits (2). Furthermore, recurrent vomiting is a gastrointestinal symptom of SPR deficiency (2). SPR deficiency can be most reliably diagnosed through analysis of cerebrospinal fluid (CSF); CSF concentrations of BH₂ and sepiapterin in SPR deficiency are significantly increased whereas those of dopamine and serotonin metabolites are decreased (2). An effect of sulfamethoxazole-trimethoprim on bipterin

levels in CSF has been reported previously: Patients with Machado-Joseph disease, an autosomal dominantly inherited neurodegenerative disorder, who were administered sulfamethoxazole-trimethoprim (400 mg sulfamethoxazole, 80 mg trimethoprim, twice daily) showed significantly increased BH₂ levels in CSF (21). Dopamine insufficiency has been reported in a child with dihydropteridine reductase (DHPR) deficiency after being given sulfamethoxazole-trimethoprim (22). DHPR is involved in recycling of BH₄ (Fig. 1A), and the observed dopamine insufficiency could be explained by a further depletion of already decreased BH₄ levels through inhibition of SPR.

To demonstrate that sulfa drugs can lower neurotransmitter levels through depletion of BH₄, we investigated whether sulfa drugs in cell culture experiments would affect catecholamine biosynthesis in the human neuroblastoma cell line SK-N-BE(2) (23). SK-N-BE(2) cells have constitutively high levels of BH₄ and excrete substantial amounts of the dopamine metabolite 3-methoxytyramine (3-MT). The identified SPR inhibitors, with the exception of sulfasalazine, at 200 μ M, decreased intracellular BH₄ and extracellular 3-MT levels (Fig. 3B). The activities in this cellular assay, again with the exception of sulfasalazine, correlated well with the IC₅₀ values measured in the enzymatic assay (Figs. 1B and 3B). For more detailed analysis, SK-N-BE(2) cells were incubated with varying concentrations of sulfathiazole, sulfamethoxazole, or sulfapyridine; the drugs decreased BH₄ levels from 0.3 to 0.05 nmol/mg protein, with IC₅₀ values of 23, 59, and 201 μ M, respectively (Fig. 3C). To examine tyrosine hydroxylase activity in drug-treated cells, extracellular L-dopa levels were measured after 24 hours of treatment with sulfathiazole, sulfamethoxazole, or sulfapyridine (Fig. 3C) (24). In these experiments, sulfamethoxazole, sulfathiazole, and sulfapyridine decreased L-dopa levels with IC₅₀ values of 31, 63, and 143 μ M, respectively. In conclusion, sulfa drugs at concentrations below their serum levels interfere with catecholamine biosynthesis through BH₄ depletion. We assume that similar effects will be observed on serotonin biosynthesis.

Our findings, together with ample clinical observations, suggest that inhibition of BH₄ biosynthesis by sulfa drugs is responsible for some of their CNS-related side effects. The incidence of these side effects will depend on the inhibitory activity of the sulfa drug toward SPR—its serum concentration during therapy, as well as its uptake into the CNS. Sulfamethoxazole reaches plasma levels of up to 600 μ M, shows good uptake into the CNS, and is a potent inhibitor of SPR in cellular assays (Fig. 1B and Fig. 3); we therefore conclude that sulfamethoxazole at these doses will affect BH₄ and catecholamine biosynthesis in the brain. The excellent CNS uptake of sulfapyridine and its good inhibitory activity against SPR explain why its CNS-related side effects are already observed at lower serum concentrations compared with other sulfa drugs also inhibiting

SPR (i.e., sulfadiazine). Furthermore, sulfa drugs not inhibiting SPR also show diminished CNS-related side effects. In early studies on the efficacy of sulfamethazine in treatment of labor pneumonia (73 patients), it was noted that “The striking difference, however, was that the patients never complained of the severe mental and physical depression which often accompanies sulfapyridine treatment” (25). CNS-related side effects have not been published for first-generation sulfonylureas tolbutamide and chlorpropamide and second-generation sulfonylurea glibenclamide. The effective serum concentration of glibenclamide is too low for SPR inhibition (Fig. 1B). However, the serum concentrations of tolbutamide and chlorpropamide are comparable to those measured for sulfa drugs. We assume that low CNS uptake of these drugs is responsible for the absence of such side effects. In fact, tolbutamide uptake in rat CNS is extremely low compared with other drugs (26, 27).

Sulfa drugs were the scaffold for the development of multiple other drugs (28). Proteins that are targeted by such drugs—for example, carbonic anhydrase and endothelin receptor—are therefore possible off-targets of sulfa drugs, and some carbonic anhydrase inhibitors (sulfonamide) or endothelin receptor antagonists (sitaxentan) cause side effects such as headache and dizziness. We could not observe inhibition of human carbonic anhydrase II, a representative isozyme of carbonic anhydrases, by sulfa drugs (IC₅₀ > 1 mM in an enzymatic assay) (table S6). Sulfamethoxazole and sulfathiazole were reported to compete with endothelin-1 for binding to endothelin receptor type A with IC₅₀ values that are within their therapeutic serum concentrations, but in functional assays sulfamethoxazole did not show activity at the highest concentrations tested (100 μ M) (table S7). These observations allow us to rule out carbonic anhydrase II but not endothelin receptor as potential additional off-targets. More generally speaking, our results do not exclude the presence of other off-targets of sulfa drugs.

Our finding that sulfa drugs interfere with BH₄-dependent neurotransmitter biosynthesis also suggests strategies for a potential improvement of PCP therapy. L-Dopa or 5-hydroxytryptophan, which are successfully used as supplements in SPR deficiency (2), might dampen some of their CNS-related side effects in high-dose sulfamethoxazole therapy. Second, sulfa drugs displaying no, or weak, SPR inhibition should be considered as alternatives to sulfamethoxazole (29). In summary, the identification of sulfa drugs as inhibitors of BH₄ biosynthesis is of relevance for understanding the history of sulfa drugs and has direct implications for their future use.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6135/987/DC1
Materials and Methods
Figs. S1 to S3
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Defining Single Molecular Forces Required to Activate Integrin and Notch Signaling

Xuefeng Wang¹ and Taekjip Ha^{1,2*}

Cell-cell and cell-matrix mechanical interactions through membrane receptors direct a wide range of cellular functions and orchestrate the development of multicellular organisms. To define the single molecular forces required to activate signaling through a ligand-receptor bond, we developed the tension gauge tether (TGT) approach in which the ligand is immobilized to a surface through a rupturable tether before receptor engagement. TGT serves as an autonomous gauge to restrict the receptor-ligand tension. Using a range of tethers with tunable tension tolerances, we show that cells apply a universal peak tension of about 40 piconewtons (pN) to single integrin-ligand bonds during initial adhesion. We find that less than 12 pN is required to activate Notch receptors. TGT can also provide a defined molecular mechanical cue to regulate cellular functions.

Cells sense and respond to the mechanical properties of the surrounding extracellular matrix (ECM) and neighboring cells. Reciprocally, cells also apply force on the ECM and transmit mechanical signals to neighboring cells. These mechanical interactions activate intracellular signaling pathways and regulate such diverse processes as cell adhesion, polarization, migration, proliferation, and differentiation (*1, 2*). As a result, by tuning bulk mechanical properties like stiffness, texture, and geometry of the substrate, researchers have gained insight into processes such as stem cell differentiation (*3*) and tumor metastasis (*4*). Single-molecule force spectroscopy has been used to study various mechanosensitive membrane receptors, including integrin, cadherin, and Notch (*5–8*). However, these approaches cannot reveal the single-molecule forces required for physiological functions because they either measure collective forces exerted through

many molecules or probe molecular unbinding or unfolding forces only. More recently, fluorescence resonance energy transfer–based force sensors were developed and inserted to target sites to monitor cellular forces (*9, 10*), but great efforts must be taken to prepare the sensors and to track and interpret the fluorescence signal. Here, we describe a platform termed tension gauge tether (TGT) that allows us to determine the single-molecule forces required for mechanical signaling in cells.

In TGT, a ligand is covalently conjugated to a tether that ruptures at a critical force, which we term “tension tolerance” or T_{tol} , and is immobilized on a solid surface through the tether (Fig. 1). Cells are plated on the surface, and membrane receptors engage with and apply tension to the ligands. If signal activation through the receptor requires a molecular tension larger than T_{tol} , the tether will rupture, abolishing signal activation. In contrast, if the required tension is smaller than T_{tol} , the tether will endure, activating the receptor-mediated signaling. By engineering a series of tethers with different T_{tol} values, the tension required for signal activation can be determined by observing receptor-regulated cell activities, which are usually much easier to detect than

single-molecule events. Because each ligand is equipped with an individual tension gauge, the measurement is independent of the receptor or ligand density.

DNA is a good candidate for use as rupturable tether in TGT because force application geometry (fig. S1) strongly affects its rupture force, with an unzipping force rupturing the DNA at a much lower force than a shearing force (*11, 12*). Albrecht *et al.* have exploited this feature to estimate the rupture force of an antigen-antibody bond relative to the rupture forces of double-stranded DNA (dsDNA) in unzipping or shear geometry (*13*). The estimated rupture force of a 21-base pair (bp) DNA is about 12 pN in the unzipping geometry and is about 56 pN in the shear geometry (*14*). Intermediate rupture forces can be obtained by applying forces through an internal position on the DNA duplex, and the theoretical T_{tol} values of the resulting tethers can be estimated (Fig. 1D and fig. S2). T_{tol} represents the force required to rupture the tether in less than 2 s when the force is applied at a constant level (*12*) [see supplementary text (*14*)].

Integrins are membrane receptors that mediate cell adhesion and sense and transduce mechanical information from the ECM into cells. Bulk traction forces applied through a collection of integrin-ligand bonds have been extensively examined (*15–18*). Here, we apply the TGT platform to probe the tension on a single integrin-ECM ligand bond required for cell adhesion (specifically, integrin $\alpha_v\beta_3$ and its ligand, cyclic RGDfK peptide). To reduce nonspecific adhesion, ligands with the DNA tether are immobilized through an Avidin-biotin linker on a glass surface passivated with polyethylene glycol (PEG). The Avidin-biotin unbinding force, ~160 pN (*19*), is much larger than the tether rupture forces used here (≤ 56 pN).

We conjugated RGDfK to nine different DNA tethers with estimated T_{tol} values of 12, 16, 23, 33, 43, 50, 54, 55, and 56 pN (fig. S2). These DNA tethers have various force application geometries but share the same length, sequence, and thermal stability. A TGT array was created by

¹Department of Physics, Center for the Physics of Living Cells and Institute for Genomic Biology University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA. ²Howard Hughes Medical Institute, Urbana, IL 61801, USA.

*Corresponding author. E-mail: tjha@illinois.edu

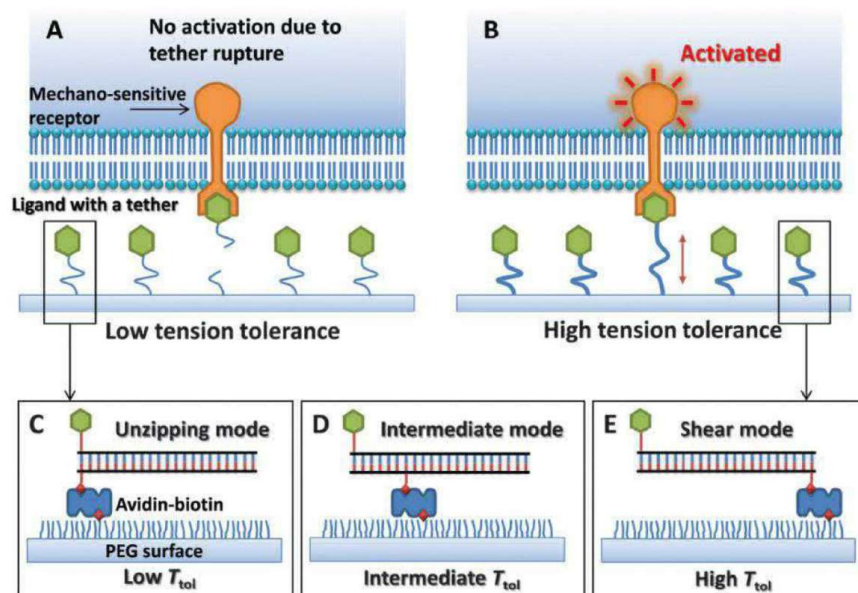


Fig. 1. Principle of TGT. A ligand for a membrane receptor is immobilized on the surface through a tether that ruptures if the tension applied by the cell through the receptor is larger than its T_{tol} . Signaling through the receptor is not activated if the tension required for activation exceeds T_{tol} and ruptures the tether (A) and is activated if T_{tol} is larger than the required tension (B). (C to E) DNA duplex helix as a tether with tunable T_{tol} values.

depositing the nine constructs as 2-mm-diameter spots at a surface ligand density of 600 molecules/ μm^2 (Fig. 2A) (see calibration in fig. S3). CHO-K1 cells were incubated on this surface for 30 min at a density of 1×10^6 cells/mL. The surface was gently rinsed, and unbound cells were removed through media exchange. Phase-contrast images were taken to measure the cell density.

We observed little cell adhesion for DNA tethers with $T_{tol} \leq 33$ pN and significant adhesion for $T_{tol} \geq 43$ pN with the cell counts increasing slightly with increasing T_{tol} (Fig. 2, B and C), suggesting that the peak tension CHO-K1 cells generate on a single RGDfK-integrin $\alpha_v\beta_3$ bond during adhesion lies mostly in the range of 33 to ~ 43 pN. To examine the effect of ligand density, we created the same TGT surface with lower ligand densities and arrived at the same tension threshold for cell adhesion (fig. S4). Other cell lines were also tested: MDA-MB-231, HeLa, HEK 293, and NIH/3T3 cells. Remarkably, all of the measured molecular tension thresholds fall within the same range, with the largest increase in cell counts occurring between a T_{tol} of 33 and 43 pN (Fig. 2D and fig. S5). In contrast, bulk traction forces normally vary among different

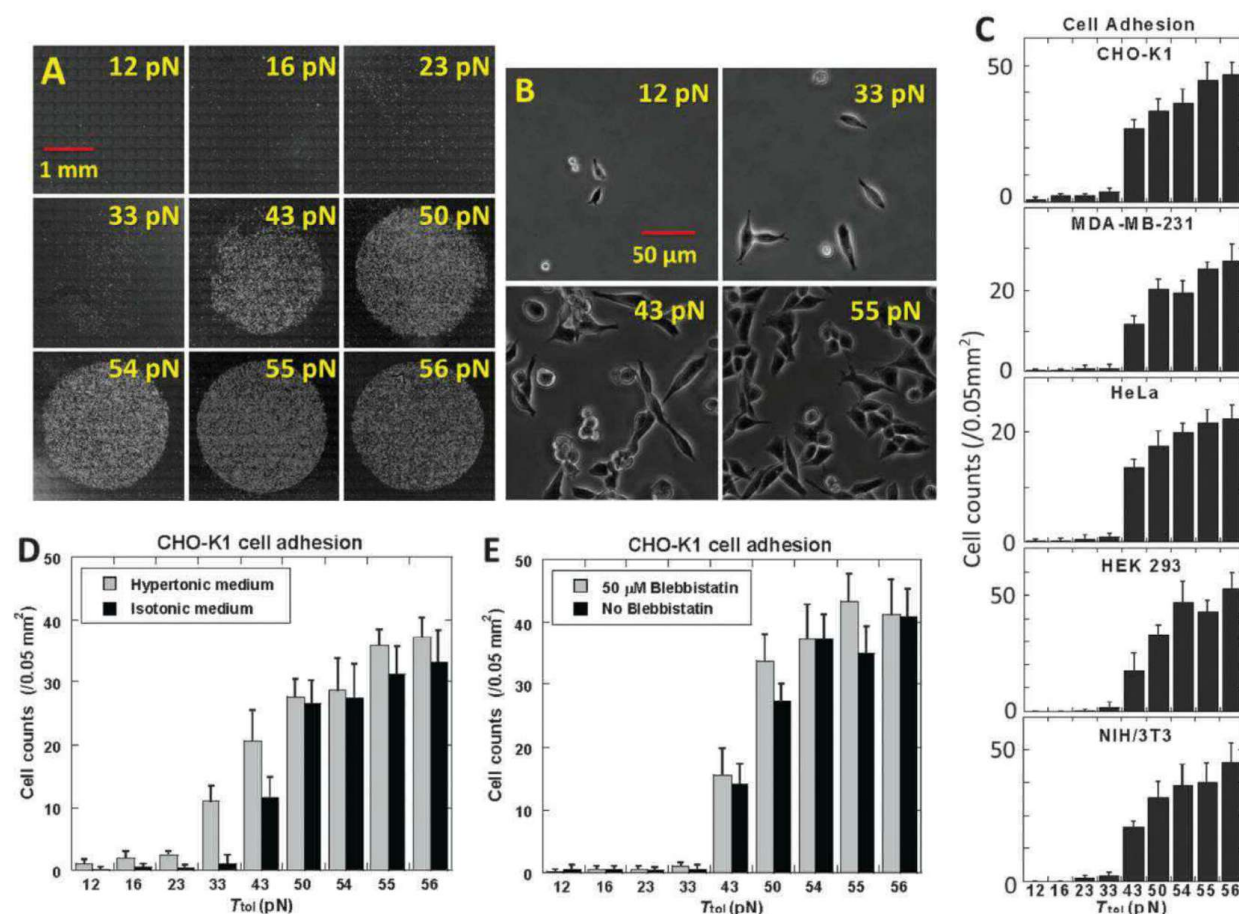


Fig. 2. Cell adhesion on TGT with RGDfK. (A) Phase-contrast images of adherent CHO-K1 cells (white dots) on circular regions coated with nine TGT constructs of RGDfK-conjugated dsDNA with the indicated T_{tol} values. (B) Zoomed-in phase-contrast images of CHO-K1 cells on four of the regions. (C)

Cell density (counts per 0.05 mm²) as a function of T_{tol} for five different cell lines as indicated. (D) Cell density versus T_{tol} for CHO-K1 cells in hypertonic versus isotonic medium. (E) Cell density versus T_{tol} for CHO-K1 cells with and without 50 μM Blebbistatin. Error bars, mean $\pm$ SD ($n = 8$ cell counting regions).

cell lines (20–22). Our observation suggests that there exists a common tension threshold value for the molecular tension generated by cells on an active integrin $\alpha_v\beta_3$ during adhesion. The same tension threshold was observed for cell seeding times as short as 5 min (figs. S6 and S7).

Using constructs that cannot be ruptured but have similar ligand accessibility as the TGTs and additional constructs with similar ligand accessibility but with different force application geometries, we could ensure that the close proximity of the ligand and biotin in the low T_{tol} constructs does not reduce ligand accessibility and cause poor cell adhesion (fig. S8). We also confirmed that the tether indeed ruptures in low T_{tol} constructs by labeling RGDfK-conjugated DNA strand with Cy3 fluorophore and observing the loss of fluorescence under the cell plating sites (figs. S9 and S10).

Because integrins are membrane proteins that are anchored to the cytoskeleton, the cell membrane and the cytoskeleton are two potential determinants of the tension threshold. We used a hypertonic medium to reduce the membrane tension and the myosin II inhibitor blebbistatin to reduce the cytoskeleton tension. The tension threshold shifted to 23 to ~33 pN for cells in the hypertonic medium (150 mM sucrose), as compared with 33 to ~43 pN in an isotonic medium (Fig. 2E and fig. S11). In contrast, the tension threshold

remained unchanged with 0 to 100 μM blebbistatin (Fig. 2E and figs. S12 and S13). Cell morphology changes confirmed the efficacy of the blebbistatin treatment (fig. S14). The data suggests that molecular tension on integrins during the initial stages of cell adhesion is primarily regulated by the membrane tension, reminiscent of a recent study that found the membrane tension to play a dominant role in restricting actin assembly and Rac activation in the leading-edge protrusion of migrating cells (23).

We next examined the formation of focal adhesions (FAs), which can occur following initial cell adhesion and spreading. FAs consist of clusters of integrins and other adaptor proteins such as vinculin, talin, and paxillin, and their formation requires a mechanical stimulus (24, 25). We asked whether the measured ~40 pN tension threshold reflects initial cell adhesion or FA formation. On a regular petri dish surface, CHO-K1 cells formed FAs and well-organized stress fibers, as detected through vinculin immunostaining and F-actin staining by phalloidin–tetramethyl rhodamine isothiocyanate, respectively (fig. S15). We seeded CHO-K1 cells for 30 min, 1 hour, and 2 hours on two TGT surfaces, one with the highest $T_{\text{tol}} = 56$ pN and the other with $T_{\text{tol}} = 43$ pN, which is just above the tension threshold for adhesion. At the 30-min seeding time, cells adhered and spread on both surfaces, but vinculin remained diffusive

and stress fibers did not form on either surface (fig. S16), confirming that the initial stages of cell adhesion and FA formation are two separate stages. Together with the observation that the threshold tension for adhesion takes effect at an early time point (5 min) of initial cell spreading, which typically lasts 15 min and is independent of talin (26), we conclude that ~40 pN tension, which is required for initial cell adhesion, acts before FA formation. We speculate that integrins may work alone without clustering in the initial stages of cell adhesion, resulting in our observed common tension threshold for different cell lines. At the 1-hour seeding time, FAs and stress fibers started to form on the 56 pN surface but not on the 43 pN surface (fig. S16). After 2 hours, FAs and stress fibers were well formed on the 56 pN surface but still not on the 43 pN surface (Fig. 3). Thus FA formation requires a molecular tension larger than that required for initial cell adhesion.

Next, we examined the molecular tension required for Notch receptor activation using a fluorescence reporter, H2B-YFP (yellow fluorescent protein). The Notch signaling pathway is responsible for intercellular communication, cell differentiation, and cell fate determination. The Notch receptor, a transmembrane protein, is activated by binding to its ligands on the neighboring cell's membrane (27). Several studies have implicated mechanical forces in Notch activation. For example, a force applied to a Notch-ligand bond may expose a cleavage site of Notch to initiate activation (28–30).

To determine the force required, we conjugated a Notch ligand DLL1 (delta-like protein 1) to a DNA strand and bovine serum albumin (BSA) to the complementary strand. DLL1-DNA (25 nM) was annealed to BSA-DNA immobilized on a glass coverslip treated with fibronectin. After blocking the surface with free BSA, we seeded CHO-K1 cells stably expressing human NOTCH1 with its intracellular domain replaced by the activator Gal4^{esn}. This cell line was also transfected with Gal4^{esn}-controlled H2B-YFP. After notch activation, H2B-YFP fluorescence was detected in the nucleus, reaching an optimal level after 2 days (31). We tested 24-bp DNA tethers, one in unzipping and the other in shear geometry with an estimated T_{tol} of 12 and 58 pN, respectively. Cells on both surfaces expressed H2B-YFP, indicating Notch activation (Fig. 4, A and B). As a control, a surface with TGT in the unzipping configuration was further treated with DNase I, which cleaves the DNA tether. As another control, DLL1-DNA conjugate was incubated with a surface coated with free BSA only (i.e., no complementary strand on the surface). On both surfaces, the YFP fluorescence level was dramatically lower (Fig. 4, C and D), ruling out the possibility that Notch is activated by non-specifically adsorbed DLL1. Other tethers with $T_{\text{tol}} = 45, 50,$ and 53 pN all resulted in Notch activation (fig. S17). We conclude that Notch activation requires either no tension or a tension below 12 pN.

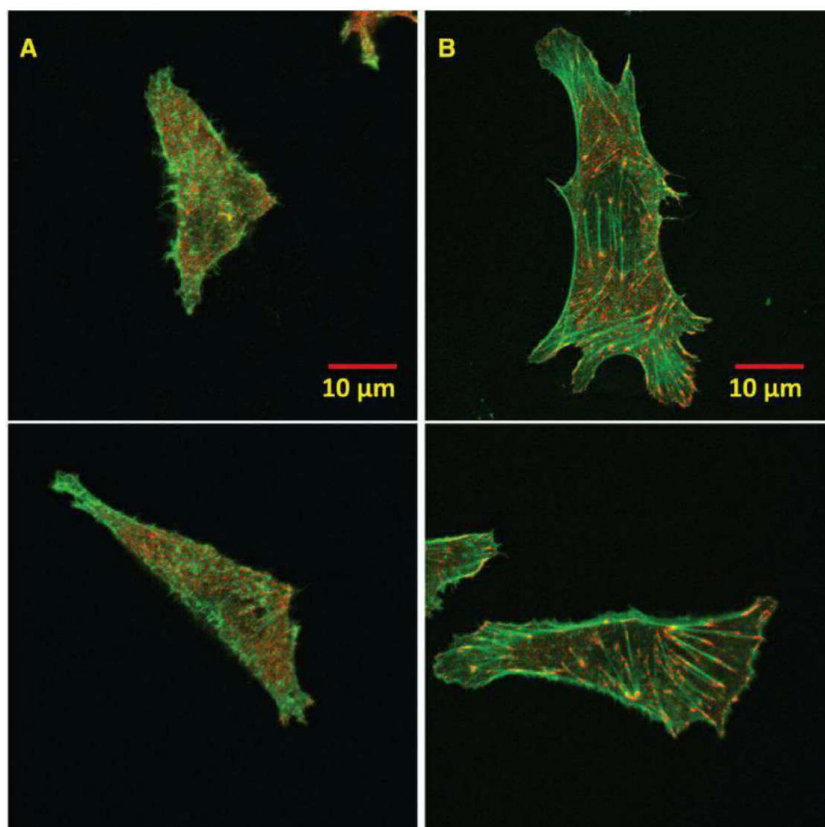


Fig. 3. Confocal fluorescent images of CHO-K1 cells. (A) A 43-pN TGT surface. (B) A 56-pN TGT surface. Actin in green and vinculin in red. Images were obtained after 2-hour cell plating. Bright green lines in (B) are stress fibers that terminate in focal adhesion complexes marked in red.

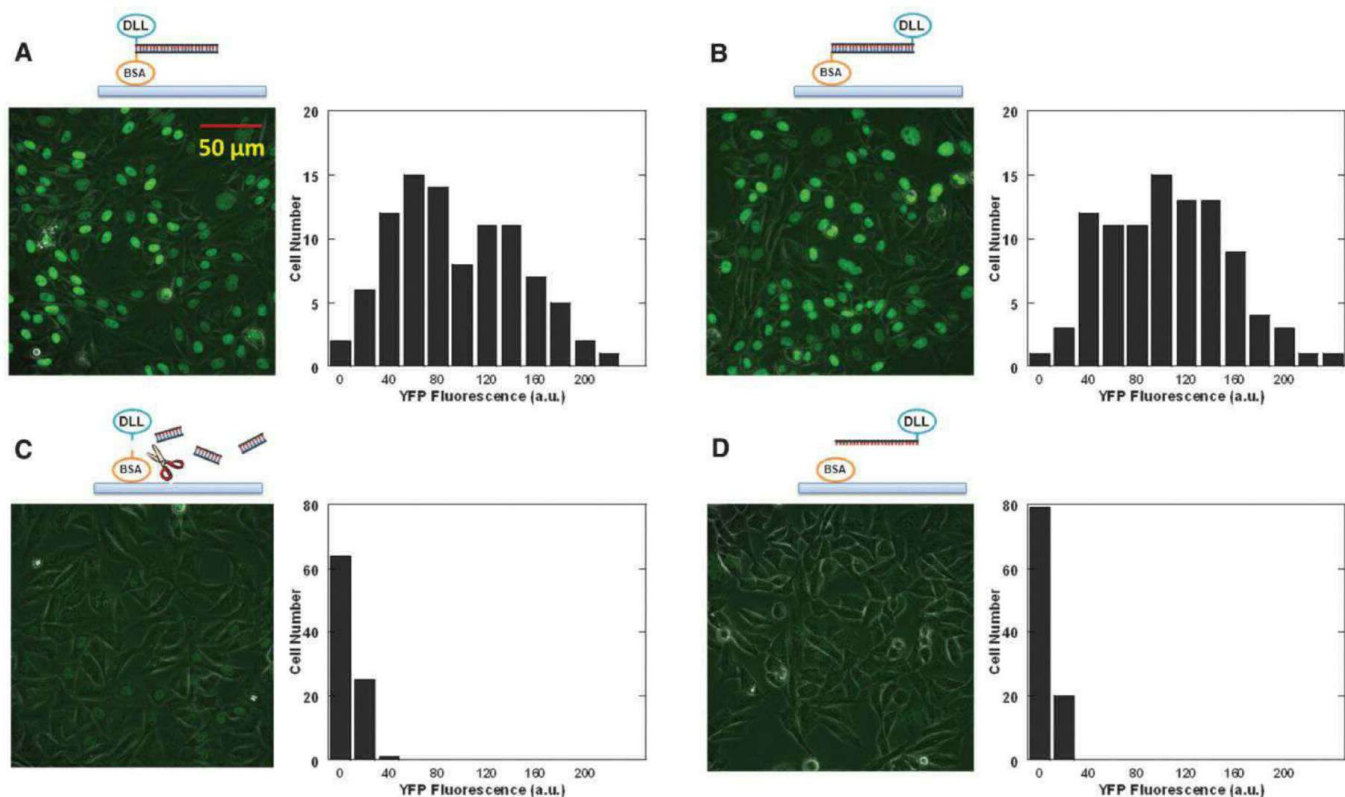


Fig. 4. H2B-YFP expression in the nucleus as a reporter of Notch activation. (A to D) YFP fluorescence images of CHO-K1 cells show nuclear fluorescence of H2B-YFP when Notch is activated. Histograms of fluorescence intensities of single cells are also shown. a.u., arbitrary unit. (A) $T_{\text{tol}} = 12$ pN.

(B) $T_{\text{tol}} = 58$ pN. (C) 12-pN TGT with DNase I digestion. (D) A control with unconjugated BSA. Here, we used BSA for TGT immobilization and surface passivation because PEG surface did not survive the 2-day period required for our Notch reporter system.

We used TGT mainly as a measurement tool here, but it can also provide a defined single-molecule mechanical cue to the cells, as we demonstrated by showing that T_{tol} values control the formation of stress fibers and FAs. It is well known that mechanical cues stemming from substrate stiffness or micropatterning can affect cancer cell or stem cell behaviors (1, 32). Tunable mechanical properties that have been examined were bulk features such as compliance, texture, and geometry. However, the sensors of mechanical cues are normally composed of single molecules, and questions remain about how a receptor as a single molecule can sense bulk properties such as stiffness. We suggest that the TGT platform provides a viable means to address these questions.

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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 to S17
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Faculty Positions Assistant and Associate Professors Center for Cell Biology & Cancer Research Albany Medical College

The Center for Cell Biology & Cancer Research at the Albany Medical College invites applications for tenure-track faculty positions at the Assistant and Associate Professor levels. Candidates' research should complement existing programs in the Center which has a strong emphasis on the tissue microenvironment with particular emphasis on model systems relevant to cancer, wound repair, and regenerative biology. We are particularly interested in applicants that utilize multi-faceted integrated approaches involving translational models to address fundamental problems in tissue function and the pathophysiology of human disease. The applicant will be expected to develop an extramurally-funded research program and participate in the teaching of graduate and medical students. Faculty will have the opportunity to be affiliated with the Cancer Genomics Center at the University of Albany East Campus. The Albany area is also home to Rensselaer Polytechnic Institute, the College of Nanoscale Science & Engineering at the University of Albany, the New York State Department of Health Wadsworth Center for Research, Taconic Farms, the State University of New York at Albany, the Albany College of Pharmacy, and General Electric Health Care Research, all of which contribute significantly to the collaborative scientific environment in the Capital District.

Qualifications include a Ph.D., DVM or M.D. degree and a demonstrated track record of excellence in research. Associate Professor candidates are expected to have an externally-funded research program. All faculty in the Center participate in the College Graduate and Medical School teaching missions. Full consideration will be given to those applications received by **July 15, 2013**. Curriculum vitae, description of research interests, and at least three letters of recommendation are required; providing copies of published papers is strongly encouraged. All materials should be submitted to: **Paul J. Higgins, Ph.D., Center for Cell Biology & Cancer Research (MC-165), Albany Medical College, 47 New Scotland Avenue, Albany, New York 12208.**

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The University of Edinburgh

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Wellcome Trust
Centre for Cell Biology WTCB

The Wellcome Trust Centre for Cell Biology

Research Group Leader Positions

The Wellcome Trust Centre for Cell Biology (WTCCB) in the School of Biological Sciences at the University of Edinburgh seeks to recruit outstanding young scientists for independent Research Group Leader positions, at the level of Career Development or Senior Research Fellows.

The WTCCB is equipped with state-of-the art facilities and instrumentation for cell biological research, including core support in microscopy, bioinformatics, proteomics and structural biology. Current research strengths at the WTCCB are in chromosome biology (including chromatin structure, epigenetics, and kinetochore function/checkpoints), subcellular organization (nuclear envelope, cytoskeleton, and mitotic/meiotic mechanisms) and RNA metabolism (non-coding RNA, microRNA, mRNA and rRNA), with an emphasis on model systems. We encourage applications in all areas complementary to our research portfolio. New investigators who apply cutting edge mass spectrometry/proteomics, super-resolution microscopy or yeast high throughput screening approaches to their research question, would be particularly welcome.

Funds are available to support successful applicants immediately for up to 3 years from commencement of their research in Edinburgh. Most group leaders within the WTCCB are supported by independent research fellowships and successful candidates would also be expected to secure independent funding.

Please apply through the University jobs site, vacancy ref 014104. Applications should include a CV (including names of three academic referees) and a brief research outline/proposal. Informal enquiries are welcome in advance and can be sent to The Search Committee Secretary, email: christine.struthers@ed.ac.uk

Applicants should ask their referees to email letters of reference to christine.struthers@ed.ac.uk by the deadline (below).

Further information about the WTCCB can be found at <http://www.wcb.ed.ac.uk>.

Details about Wellcome Trust Career Development (Sir Henry Dale) and Senior Research Fellowships can be found at:

<http://www.wellcome.ac.uk/Funding/Biomedical-science/Funding-schemes/Fellowships/Basic-biomedical-fellowships/WTDV031823.htm>

and

<http://www.wellcome.ac.uk/Funding/Biomedical-science/Funding-schemes/Fellowships/Basic-biomedical-fellowships/WTDO04442.htm>

Closing date: 12 June 2013.

Interviews for short-listed candidates are likely to be held in mid-late August 2013.

Committed to Equality and Diversity

The University of Edinburgh is a charitable body, registered in Scotland, with registration number SC005336.

www.ed.ac.uk/jobs

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POSITIONS OPEN

SCIENTIST: PROTEIN BIOCHEMISTRY

The Fraunhofer USA Center for Molecular Biotechnology in Newark, Delaware is seeking a highly motivated Ph.D. level **Protein Biochemist** to work in the Protein Biochemistry group to assist in development and optimization of scalable purification processes for membrane associated proteins, poorly soluble proteins, and virus-like particles produced in our plant-based expression platform. The Center focuses on the development of subunit vaccines, protein therapeutics, and reagents.

The position is for a bench level scientist who will fill an interdisciplinary role working on many aspects of the development process. Roles will include investigation of purification and characterization of poorly soluble targets, integral membrane proteins, and enveloped virus like particles. The candidate will be expected to research, develop, and implement new technologies/methodologies for the Protein Biochemistry group as well as for possible transfer to QC or GMP bioprocess facilities.

A solid understanding of protein chemistry, biochemistry, and protein analytical techniques is required. The selected candidate must have strong organizational and communication skills. The position requires extensive previous purification experience with membrane-associated proteins, enveloped virus like particles, and/or poorly soluble proteins and a track record of achievement in this area.

A Ph.D. degree in Protein Biochemistry, Biotechnology, or in a related discipline with at least four years laboratory experience and a demonstrated background as described are required.

Fraunhofer USA offers competitive salaries and benefit packages and is an Equal Opportunity Employer.

To apply, please visit **website: <https://home2.ecase.adp.com/recruit/?id=6926692>**.

For more information about Fraunhofer USA CMB please visit our **website: <http://www.fraunhofer-cmb.org/index.htm>**.

TENURE-TRACK ASSISTANT/ASSOCIATE PROFESSOR

The Department of Physiology at Wayne State University (WSU) School of Medicine invites applications for a tenure-track Assistant/Associate Professor position. Major areas of interest include cellular, molecular, and systems approaches to investigations related to contemporary physiology and pathophysiology. Special consideration will be given to investigators who complement existing strengths of the Department (**website: <http://physiology.med.wayne.edu>**).

Candidates are expected to establish active extramurally funded research programs and participate in teaching medical and graduate students. Startup packages and salaries are highly competitive. Candidates must hold Ph.D., M.D., or equivalent and can apply with curriculum vitae, detailed research plan, and names/contact information of three references to **e-mail: WSUPhysiologyFacultySearch@med.wayne.edu**. Review of applications will begin after July 1, 2013 and continue until position is filled.

WSU offers 350 academic programs through 14 schools and colleges to over 31,000 students in the Detroit Midtown area. WSU School of Medicine is a state-of-the-art research environment, and rated by the Carnegie Foundation in the top third of all U.S. Research Institutions. The Detroit Metro area is home to four million people and a hub to many of the nation's high-tech industries.

WSU is an Equal Opportunity/Affirmative Action Employer.

MARKETPLACE

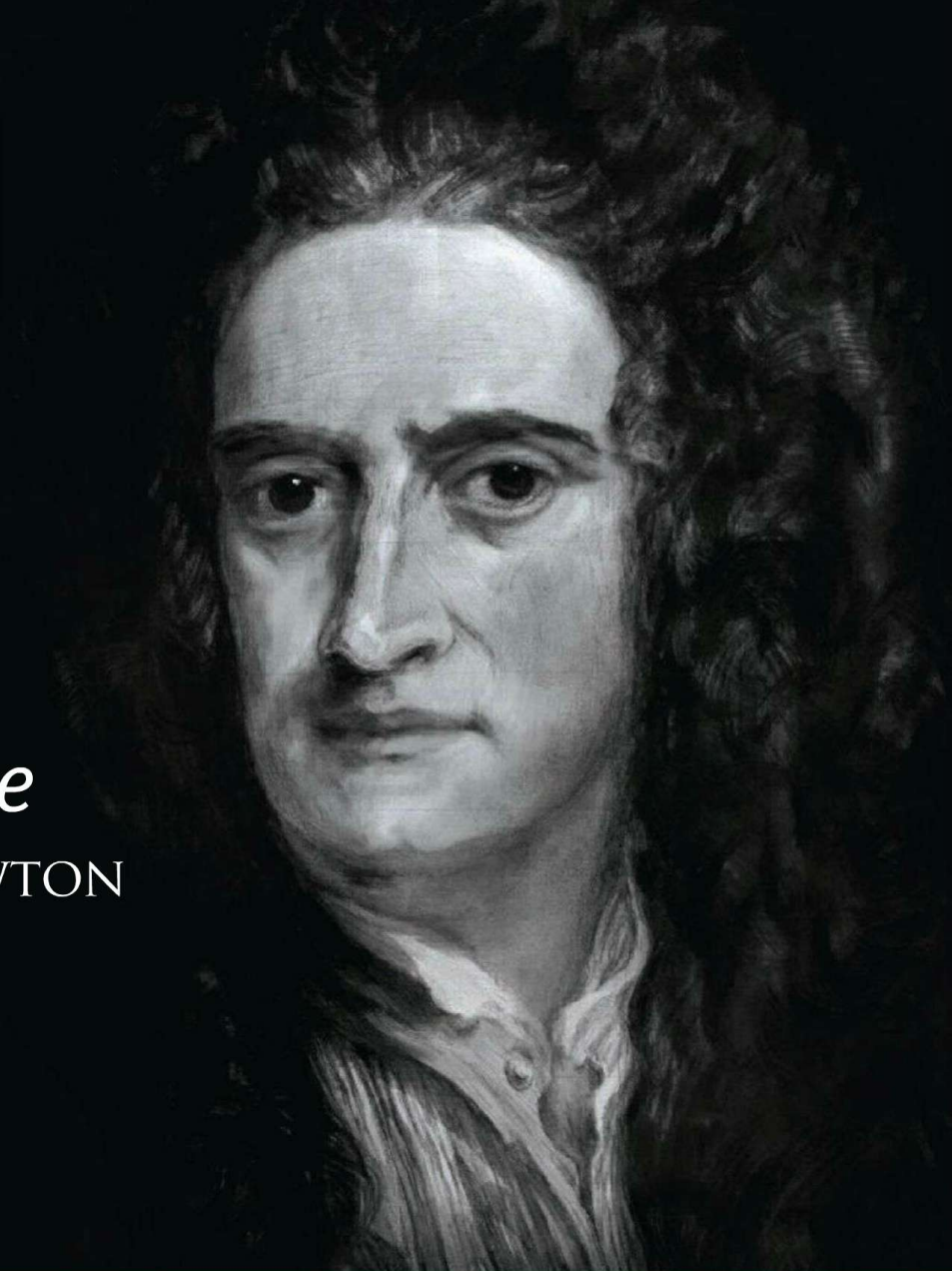
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A large, detailed portrait of Sir Isaac Newton, showing him with long, curly hair and a serious expression, wearing a dark coat over a white shirt.

Science

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SIR ISAAC NEWTON

Sir Isaac Newton's contribution to science can only be described as unique. Over his lifetime, Newton offered insights into physics, mathematics, natural philosophy, and even alchemy, and is now considered by many to be one of the greatest scientists who ever lived. In 1687, the publication of his *Philosophiæ Naturalis Principia Mathematica* was an influential landmark in scientific thinking that defined the principles of universal gravitation and the laws of motion—setting the foundation that scientists would turn to for over 300 years.

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